(54) Title: APOPTOSIS RELATED PROTEIN BCL-Y, AND METHODS OF USE THEREOF

(57) Abstract

The present invention is directed to an isolated Bcl-Y protein, nucleotide sequences coding for and regulating expression of the protein, antibodies directed against the protein, and recombinant vectors and host cells containing the genetic sequences coding for and regulating the expression of the protein sequence. The invention is also directed to genomic DNA, cDNA, and RNA encoding the Bcl-Y protein sequence and to corresponding antisense RNA sequences. Antibodies can be used to detect Bcl-Y in biological specimens, including, for example, human tissue samples. The present invention is further directed to methods of treating degenerative disorders characterized in inappropriate cell proliferation or inappropriate cell death. The present invention is further directed to methods for diagnosing degenerative disorders characterized in inappropriate cell proliferation or inappropriate cell death, as well as methods for monitoring the progress of such degenerative disorders.

APOPTOSIS RELATED PROTEIN BCL-Y, AND METHODS OF USE THEREOF

Cross Reference to Related Applications

This application is a continuation-in-part of co-pending United States application Serial Number 08/287,427, filed 9 August 1994.

Field of the Invention

The present invention relates generally to the field of cell physiology, and more particularly, to apoptosis. Even more particularly, the present invention is related to the novel apoptosis associated protein Bcl-Y; to nucleotide sequences encoding Bcl-Y; to products and processes involved in the cloning, preparation and expression of genes and nucleotide sequences encoding Bcl-Y; to antibodies with specificity to Bcl-Y; and to diagnostic and therapeutic uses of the above.

Background of the Invention

Apoptosis refers to cell suicide that proceeds by an active, physiological process (Kerr, J.F., et al., Br. J. Cancer 26:239-257 (1972); Wyllie, A.H., Nature 284:555-556 (1980)). Cells that die by apoptosis undergo characteristic morphological changes, including cell shrinkage, and nuclear condensation and fragmentation. Apoptosis plays an important role in developmental processes, including morphogenesis, maturation of the immune system, and tissue homeostasis whereby cell numbers are limited in tissues that are continually renewed by cell division (Ellis, R.E., et al., Annu. Rev. Cell. Biol. 7:663-698 (1991); Oppenheim, R.W., et al., Neurosci. 14:453-501 (1991); Cohen, J.J., et

al., *Annu. Rev. Immunol.* *10*:267-293 (1992); Raff, M.C.,
Nature *356*:397-400 (1992)). While the cell death program
can be activated through a diversity of normal physiological
stimuli, cells also die by apoptosis following a variety of
5   non-physiological insults, including irradiation and
exposure to DNA damaging drugs (Eastman, A., *Cancer Cells*
2:275-280 (1990); Dive, C., *et al.*, *Br. J. Cancer* *64*:192-196
(1991); Lennon, S.V., *et al.*, *Cell prolif.* *24*:203-214
(1991)).

10   In addition to its role in developmental processes,
apoptosis is an important cellular safeguard against
tumorigenesis (Williams, G.T., *Cell* *65*:1097-1098 (1991);
Lane, D.P. *Nature* *362*:786-787 (1993)). Defects in the
apoptotic pathway may contribute to the onset or progression
15   of malignancies. Under certain conditions, cells undergo
apoptosis in response to forced expression of oncogenes, or
other genes that drive cell proliferation; (Askew, D., *et
al.*, *Oncogene* *6*:195-1922 (1991); Evan, G.I., *et al.*, *Cell*
69:119-128 (1992); Rao, L., *et al.*, *Proc. Natl. Acad. Sci.*
20   *USA* *89*:7742-7746 (1992); Smeyne, R.J., *et al.*, *Nature*
363:166-169 (1993)). The potential importance of apoptosis
as a defense against tumorigenesis is underscored by the
observation that suppression of the apoptotic pathway(s), by
a variety of genetic lesions, occurs frequently in a broad
25   range of human tumors. As a specific example, loss of the
p53 tumor suppressor gene function, either through deletion
or mutation, occurs in more than 50% of human cancers
(Levine, A.J., *et al.*, *Nature* *351*:453-456 (1991); Lane, D.P.
Nature *362*:786-787 (1993)). The p53 protein is required for
30   efficient induction of apoptosis by DNA damaging agents
(including irradiation and most chemotherapeutic drugs)
(Clark, A.R., *et al.*, *Nature* *362*:849-852 (1993); Lowe, S.W.,
et al., *Cell* *74*:957-967 (1993a); Lowe, S.W., *et al.*, *Nature*
362:847-849 (1993b)). This activity is likely to be at
35   least part of the tumor suppressor function of p53. Cells
that suffer extensive, and potentially carcinogenic, genetic

damage may self destruct via a p53 dependent mechanism thereby reducing the potential for tumorigenesis (Lane, D.P. *Nature 362*:786-787 (1993)).

In addition to cancer, deregulation of apoptosis may contribute to a number of other human diseases. A variety of degenerative disorders may involve aberrant apoptosis, resulting in premature or inappropriate cell death (Barr, P.J., *et al.*, *Biotechnology 12*:487-493 (1994)). Productive infection by certain viruses may depend on suppression of host cell death by anti-apoptotic viral gene products (Rao, L., *et al.*, *Proc. Natl. Acad. Sci. USA 89*:7742-7746 (1992); Ray, C.A., *et al.*, *Cell 69*:597-604 (1992); White, E., *et al.*, *Mol. Cell. Biol. 12*:2570-2580 (1992); Vaux, D.L., *et al.*, *Cell 76*:777-779 (1994), and inhibition of apoptosis can alter the course (i.e. lytic vs. latent) of viral infection; Levine, B., *et al.*, *Nature 361*:739-742 (1993)). Widespread apoptosis of T lymphocytes triggered by HIV infection may, at least in part, be responsible for the immune system failure associated with AIDS (Gougeon M., *et al.*, *Science 260*:1269-1270 (1993)). Therefore, from the perspective of pharmaceutical development, it would be desirable to identify or develop agents that either activate, or suppress, apoptosis depending on the clinical setting.

The bcl-2 gene product has been intensively studied as a potent suppressor of apoptotic cell death. The bcl-2 gene was originally identified at the t(14:18) translocation breakpoint that occurs frequently in human B cell follicular lymphomas (Bakhshi, A., *et al.*, *Cell 41*:899-906 (1985); Cleary, M.L., *et al.*, *Proc. Natl. Acad. Sci. USA 82*: (1985); Tsujimoto, Y., *et al.*, *Science 229*:1390-1393 (1985)). This translocation results in the constitutive activation of bcl-2 gene expression due to juxtaposition with the immunoglobulin heavy chain locus. Bcl-2 functions as an oncogene in this disease by inappropriately suppressing apoptosis that would normally limit the accumulation of these cells (McDonnell, T.J., *et al.*, *Cell 57*:79-88 (1989);

Hockenbery, D., et al., *Nature 348*:334-336 (1990)).
Consequently, B cells accumulate during the indolent stage
of the lymphoma due to their failure to die rather than by
uncontrolled proliferation.

5   Targeted gene disruption experiments indicate that one
important normal function of Bcl-2 is to contribute to the
maintenance and survival of the mature immune system
(Nakayama, K., et al., *Science 261*:1584-1588 (1993); Veis,
D.J., et al., *Cell 75*:229-240 (1993)). However, the anti-
10 apoptotic activity of Bcl-2 is not restricted to B cells. A
large number of studies have demonstrated that ectopic Bcl-2
expression can suppress apoptosis triggered by diverse
stimuli in a multitude of cell lineages (Vaux, D.L., et al.,
Nature 335:440-442 (1988); Sentman, C.L., et al., *Cell
15 67*:879-888 (1991); Strasser, A., et al., *Cell 67*:889-899
(1991); Hockenbery, D.M., et al., *Cell 75*:241-251 (1993)).
Bcl-2 blocks cell death induced by growth factor withdrawal,
DNA damage, oncogene expression, oxidative stress, viral
infection. The ability of Bcl-2 to block apoptosis in
20 virtually every system suggests that Bcl-2 is closely
connected with the machinery that actually carries out the
death program. This view is further supported by the
conservation of Bcl-2 function across species. The ced9
gene in the nematode c. Elegans functions to suppress
25 programmed death in certain cell lineages of the developing
worm (Ellis, H.M., et al., *Cell 44*:817-829 (1986)). Ced9
appears to be a functional homologue of Bcl-2, since Bcl-2
can complement ced9 in transgenic worms (Vaux, D.L., et al.,
Science 258:1955-1957 (1991)). Bcl-2 can also function in
30 insect cells as demonstrated by the ability of Bcl-2 to
suppress apoptosis induced by Baculovirus infection
(Alnemri, E.S., et al., *Proc. Natl. Acad. Sci. USA
89*:7295-7299 (1992)).

The molecular mechanism whereby Bcl-2 operates to block
35 cell death is poorly understood. Bcl-2 is a 25 kD membrane
associated protein that has been detected in mitochondrial

membranes, the nuclear envelope, and the endoplasmic
reticulum (Chen-Levy, et al., *Mol. Cell. Biol.* 9:701-710
(1989); Hockenbery, D., et al., *Nature* 348:334-336 (1990);
Jacobson, et al., *Nature* 361:365-369 (1993)). Bcl-2 has
5 been found to physically associate with the ras related
protein r-Ras (Fernandez-Sarabia, et al., *Nature* 366:274-275
(1993)). However, the biological significance of this
interaction is still uncertain. Several lines of evidence
have suggested that Bcl-2 functions in an anti-oxidant
10 pathway, although it is not clear whether this is a direct
or indirect activity of Bcl-2 (Hockenbery, D.M., et al.,
Cell 75:241-251 (1993); Kane, D.J., et al., *Science*
262:1274-1277 (1993); Butte, T.M., et al., *Immunol. Today*
15:7-10 (1994)).

15   Additional cellular genes that exhibit significant
sequence homology with Bcl-2 have recently been identified
and, where tested, these genes appear also to function as
regulators of apoptotic cell death. One Bcl-2 relative,
Bcl-X, was isolated by low stringency DNA hybridization to
20 the Bcl-2 gene (Boise, L.H., et al., *Cell* 74:597-608
(1993)). The Bcl-X RNA is differentially spliced to
produce a long form, termed Bcl-X_L, and a shorter form, Bcl-
X_S, bearing a short internal deletion. Bcl-X_L functions to
suppress cell death, much like Bcl-2, whereas the deleted
25 form, Bcl-X_S, can inhibit protection by Bcl-2 and may
function as a "dominant negative" species. A second Bcl-2
relative, Bax, was identified biochemically as protein found
in co-immunoprecipitates with Bcl-2 (Oltvai, Z.N., et al.,
Cell 74:609-619 (1993)). Isolation of the corresponding
30 cDNA revealed that the Bax protein shows substantial
sequence homology to Bcl-2. Bax forms heterodimers with
Bcl-2 and appears to induce apoptosis and function as a
negative regulator of Bcl-2 function. Ectopic expression
of Bax was shown to block the protection against apoptosis
35 afforded by Bcl-2 expression.

Two additional cellular Bcl-2 relatives, Mcl and A1
(Kozopas, K.M., et al., *Proc. Natl. Acad. Sci. USA*
90:3516-3520 (1993); Lin, E.Y., et al., *J. Immunol.*
151:1979-1988 (1993)) were originally isolated as mRNAs
induced in response to specific stimuli: phorbol ester
induced differentiation of myeloid leukemia cells (Mcl) and
GM-CSF treatment of murine bone marrow cells (A1). It is
not yet known whether either Mcl or A1 can modulate
apoptosis.

In addition to these cellular Bcl-2 relatives, a number
of Bcl-2 homologues encoded by DNA viruses have been
identified. The Epstein-Barr virus BHRF-1 gene product was
noted to contain sequence homology to Bcl-2 and has
subsequently been shown to function as a suppressor of
apoptosis (Henderson, et al., *Proc. Natl. Acad. Sci. USA*
90:8479-8488 (1993)). Likewise, the African swine fever
virus LMW5-HL gene encodes a protein structurally similar to
Bcl-2 (Neilan, J.G., et al., *J. Virol. 67*:4391-4394 (1993)).
The Adenovirus E1b 19kD protein appears to be functionally
equivalent to Bcl-2, although the primary sequence homology
is quite limited (White, E., et al., *Mol. Cell. Biol.*
12:2570-2580 (1992)). It is likely that these genes
function to ensure replication of viral DNA by preventing
apoptosis of the infected cell. The finding that unrelated
DNA viruses have evolved genes that apparently function to
mimic the action of Bcl-2, supports the conclusion that Bcl-
2 represents an important apoptosis regulator.

The five known cellular Bcl-2 related genes, where
analyzed, have distinct patterns of expression and thus may
function in different tissues. The cell death program is in
place in all tissues and may be regulated by different Bcl-2
related genes. While Bcl-2 expression is required for
maintenance of the mature immune system, it is desirable to
identify other genes which may govern apoptotic cell death
in other lineages.

Summary of the Invention

The present inventor has surprisingly discovered a novel composition of matter which has been isolated and characterized, and which is described in a number of embodiments herein. Referred to herein as "Bcl-Y," it appears to be a member of the Bcl-2 family, and can, *inter alia*, induce apoptosis in cells and function as a negative regulator of Bcl-2 function in cells. Isolation of a full length human Bcl-Y cDNA revealed that the deduced Bcl-Y amino acid sequence shares the most homology with Bcl-2, with additional regions of homology extending outside of domains I and II. Bcl-Y mRNA was detected in all human tumor cell lines examined, and appears to be widely expressed in primary human tissues. In view of its widespread expression and significant homology to Bcl-2, Bcl-Y is an important regulator of apoptosis in human tissues and/or tumor cells.

Bcl-Y by itself accelerates apoptosis when expressed in FL5.12 cells, an IL-3 dependent murine cell line that undergoes apoptosis upon cytokine withdrawal (Hockenbery, D., et al., Nature 348:334-336 (1990)). Also, when Bcl-Y is expressed in FL5.12 cells that are over-expressing an exogenous Bcl-2 clone, the presence of Bcl-Y reduces the protective effect of Bcl-2 under conditions designed to induce apoptosis in those cells.

The present invention thus relates to an apoptosis associated protein Bcl-Y, products and processes involved in the cloning, preparation and expression of genes for Bcl-Y; antibodies with specificity to Bcl-Y; and nucleotide probes corresponding to the Bcl-Y nucleotide sequence or portions thereof. The Bcl-Y polypeptide is useful for producing antibodies thereto. The antibodies and probes are useful for detecting and isolating Bcl-Y in biological specimens including for example, cells from all human tissues including heart tissue, lung tissue, tumor cells, brain

tissue, placenta, liver, skeletal muscle, kidney, and pancreas.

The present invention further relates to species homologs and viral homologs of Bcl-Y.

5   The present invention relates to the identification, characterization and sequencing of cDNAs and genomic fragments which encode the Bcl-Y that is present in human cells.

According to the present invention, there are provided

10   genetic sequences encoding Bcl-Y. The instant invention also provides for expression vectors containing such genetic sequences, hosts transformed with such expression vectors, and methods for producing the genetically engineered or recombinant Bcl-Y.

15   The present invention also provides antibodies which specifically recognize Bcl-Y.

The Bcl-Y cDNA and recombinant protein are useful for making antibodies which specifically recognize Bcl-Y. Such antibodies are useful for detecting and isolating Bcl-Y in a

20   biological specimen. The present Bcl-Y protein is also useful as a regulator of apoptosis.

A small cDNA from the Namalwa, ATCC CRL 1432, human B-cell lymphoma cell line, and from the human leukemia cell line U937 ATCC CRL 1593, has been isolated. The amino acid

25   sequence of the Bcl-Y protein shares sequence homology with Bcl-2, with additional regions of homology extending outside of domains I and II.

The present invention further relates to a method for isolating Bcl-Y partial clones using polymerase chain

30   reaction (PCR) cloning, from diverse human tumor cell lines.

The present invention is further directed to methods for inducing or suppressing apoptosis in individuals suffering from degenerative disorders characterized by inappropriate cell proliferation or inappropriate cell

35   death, respectively. Degenerative disorders characterized by inappropriate cell proliferation include, for example,

inflammatory conditions, cancer, including lymphomas, genotypic tumors, etc. Degenerative disorders characterized by inappropriate cell death include, for example, autoimmune diseases, acquired immunodeficiency disease (AIDS), cell death due to radiation therapy or chemotherapy, etc.

The present invention also relates to methods for detecting the presence of Bcl-Y protein, as well as methods directed to the diagnosis of degenerative disorders, which disorders are associated with an increased or decreased level of expression of Bcl-Y, as compared to the expected level of Bcl-Y expression in the normal cell population.

The present invention is further directed to methods for monitoring the progress of degenerative disorders associated with increased or decreased levels of expression of Bcl-Y, by monitoring Bcl-Y expression.

The present invention also relates to methods for determining whether a disease/degenerative disorder is linked to abnormal Bcl-Y expression, as well as methods for determining the effect of over expression or loss of expression of Bcl-Y in animal models such as transgenic mice and/or homozygous null mice. Methods for determining whether a disease/degenerative disorder is linked to abnormal Bcl-Y expression include analyzing Bcl-Y expression in diseased tissue as compared to normal tissue by for example, Northern and/or Western blots, as well as by other assay methods readily chosen and employed by those of ordinary skill in the art.

The present invention relates to hybrids of Bcl-Y for therapeutic use.

The present invention also relates to methods for modulating apoptotic effects by administering the present Bcl-Y protein, mutant protein or hybrids to an individual suffering from a degenerative disorder characterized by inappropriate cell proliferation or inappropriate cell death in order to stabilize inappropriate cell proliferation (i.e., induce apoptosis) or stabilize inappropriate cell

death (i.e., suppress apoptosis), respectively, and/or in
either case to restore normal cell behavior. Fig. 8
illustrates levels of Bcl-Y mRNA expressed in a variety of
healthy adult tissues.

The present invention further relates to functional
equivalents including functional fragments of Bcl-Y
including, for example, peptides of Bcl-Y such as Domain I
and Domain II, and other regions of homology recognized by
the present inventor between Bcl-Y and other apoptosis
related proteins including Bcl-2 and Bax.

The present invention is also directed to nucleotide
probes which can be used to determine the presence of Bcl-Y
as well as to identify and isolate homologs including
species homologs and viral homologs.

Description of the Figures

Figure 1. Conservation of domains I and II among Bcl-2
family members.

(A). Amino acid sequence homology between previously known
Bcl-2 homologues. Degenerate PCR primers (BD2 and BU3),
corresponding to conserved domains I and II (boxed), are
shown at bottom. ASFV and EBV refer to the African swine
fever virus LMW5-HL and Epstein-Barr virus BHRF1 genes,
respectively. The numbers at the far right refer to the
amino acid position number in the corresponding proteins.
(B). Conceptual translation of the Bcl-Y fragment isolated
by PCR. The Bcl-Y PCR product obtained with primers BD2 and
BU3 is shown on the top line. The underlined residues
correspond to the primers used in PCR. The shaded, bold
faced residues are sequence elements highly conserved among
the previously identified Bcl-2 relatives.

Figure 2. Expression of the Bcl-Y RNA in tumor cell lines.
Northern blot analysis was performed using mRNA prepared
from the indicated tumor cell lines. The blots were

hybridized with a 32-P labeled probe derived from the Bcl-Y PCR product. RNA size markers (Gibco/BRL) were visualized by ethidium bromide staining.

Figure 3. Bcl-Y clones isolated from Jurkat cell cDNA. A lambda cDNA library was screened with a probe derived from the Bcl-Y PCR product (hatched box, top) and three clones were obtained (clones 4, 10, 11). Lamda clone 11 encompasses the entire Bcl-Y open reading frame (open and hatched box). Additional 5' sequence was obtained by PCR using the 5' RACE method. A composite Bcl-Y cDNA, derived from these overlapping clones, is shown at the bottom.

Figure 4. DNA sequence and conceptual translation of a composite Bcl-Y cDNA. The initiating methionine codon is shown in bold face, and is preceded by stop codons (underlined) in all three reading frames.

Figure 5. Homology of Bcl-Y to Bcl-2. The three regions of Bcl-Y with the highest homology to Bcl-2 are shown. The homology and alignments were performed by the BLASTP program. Boxed, shaded residues indicate identity, lightly shaded residues indicate conservative changes.

Figure 6. Alignment of Bcl-Y with the Bcl-2 family. The amino acid positions of the various Bcl-2 relatives are indicated. Boxed, shaded residues are identical in at least 4 of the 8 Bcl-2 relatives. Lightly shaded residues indicate coservative amino acid changes. The hydrophobic C-terminal, putative membrane localization sequences, are underlined.

Figure 7. Expression of the Bcl-Y protein.
(A). In vitro translation of 35-S labeled Bcl-Y in rabbit reticulocyte lysate. Luciferase was used as a positive control for the translation reactions; "no DNA" indicates a

reaction performed with no DNA template. Proteins were
resolved by SDS gel electrophoresis and visualized by
autoradiography. The gel mobilities of pre-stained protein
molecular weight markers (Amersham) are shown.

5 (B). Expression of Bcl-Y in transfected cells. Plasmids
expressing the indicated hemagglutinin (HA) epitope-tagged
proteins were transfected into either COS7 or NIH3T3 cells.
The expression vector Rc/CMV was transfected as a negative
control. Proteins were detected 48 hrs after transfection
10 by Western blot of cell lysates with the anti-HA monoclonal
antibody 12CA5 (Boehringer Mannheim).

Figure 8. Expression of Bcl-Y in human tissues. A human
multiple tissue northern blot was hybridized sequentially
15 with 32-P labeled probes derived from Bcl-Y, Bcl-2, Bax and
ß-actin cDNA sequences.

Figure 9. Effect of Bcl-y expression on viability of FL5.12
cells following IL-3 withdrawal.

20 **(A)** Viability of FL5.12 clones expressing HA-Bcl-y after
withdrawal of IL-3. Cell viability was measured at the
indicated times following cytokine withdrawal. Each data
point represents the average of duplicate cultures assayed
in parallel. Viability assays were repeated several times
25 and results of a representative experiment are shown. Neo
is a G418-resistant (G418^r) FL5.12 clone transfected with a
plasmid encoding neomycin resistance only; yb2, yb3, and
yb4 are individual G418^r FL5.12 clones that express HA-Bcl-
y.

30 **(B)** Expression of HA-Bcl-y in FL5.12 cells. The HA-Bcl-y
protein (~29 kD) was detected by Western blot analysis of
extracts prepared from the indicated cell lines, with the
HA-specific 12CA5 antibody. An asterix denotes non-specific
bands.

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Figure 10. Effect of Bcl-Y expression in FL5.12 cell populations also expressing Bcl-2.

(A) Viability of FL5.12 cell populations expressing Bcl-2 in combination with either HA-Bcl-Y or HA-Bax following withdrawal of IL-3. Drug resistant cells that arose after infection of Bcl-2 expressing FL5.12 cells with retroviruses expressing HA-Bcl-Y, HA-Bax, or puromycin alone, were pooled and analyzed as cell populations. The neo/puro population is a G418^r FL5.12 cell line that does not express exogenous Bcl-2, infected with the puromycin-encoding retrovirus vector alone.

(B) Expression of HA-Bcl-Y, HA-Bax, and Bcl-2 in FL5.12 cell populations. Extracts were prepared from the indicated cell populations and HA-tagged proteins (left) and exogenous human Bcl-2 (right) were detected by Western blot analysis.

Figure 11. Effect of Bcl-Y expression in FL5.12 clones also expressing Bcl-2.

(A) Viability of FL5.12 clones expressing Bcl-2 in combination with either HA-Bcl-Y or HA-Bax following withdrawal of IL-3.

(B) Expression of HA-tagged proteins in FL5.12 clones. HA-tagged proteins (left) and exogenous human Bcl-2 (right) were detected by Western blot analysis.

Detailed Description of the Invention

I.   Definitions

To aid in the understanding of the specification and the claims, the following definitions are provided:

Gene. By the term "gene" is intended a DNA sequence which encodes through its template or messenger RNA a sequence of amino acids characteristic of a specific

peptide. Further, the term includes intervening, non-coding regions, as well as regulatory regions, and can include 5' and 3' ends.

5   ***Gene sequence.*** The term "gene sequence" is intended to refer generally to a DNA molecule. As used herein, the terminology is meant to include both a DNA molecule which contains a non-transcribed or non-translated sequence. The term is further intended to include any combination of
10 gene(s), gene fragment(s), non-transcribed sequence(s), or non-translated sequence(s) which are present on the same DNA molecule.

       The present sequences may be derived from a variety of
15 sources including DNA, cDNA, synthetic DNA, RNA, or combinations thereof. Such gene sequences may comprise genomic DNA which may or may not include naturally occurring introns. Moreover, such genomic DNA may be obtained in association with promoter regions or poly A sequences. The
20 gene sequences, genomic DNA or cDNA may be obtained in any of several ways. Genomic DNA can be extracted and purified from suitable cells by means well known in the art. Alternatively, mRNA can be isolated from a cell and used to produce cDNA by reverse transcription or other means.
25

       cDNA. The term "cDNA" is meant complementary or copy DNA produced from an RNA template by the action of RNA-dependent DNA polymerase (reverse transcriptase). Thus a "cDNA clone" means a duplex DNA sequence complementary to an
30 RNA molecule of interest, carried in a cloning vector. This term includes genes from which the intervening sequences have been removed.

       Recombinant DNA. By the term "recombinant DNA" is
35 meant a molecule that has been recombined by *in vitro* splicing cDNA or a genomic DNA sequence.

Cloning. By the term "cloning" is meant the use of *in vitro* recombination techniques to insert a particular gene or other DNA sequence into a vector molecule. In order to successfully clone a desired gene, it is necessary to employ

5 methods for generating DNA fragments, for joining the fragments to vector molecules, for introducing the composite DNA molecule into a host cell in which it can replicate, and for selecting the clone having the target gene from amongst the recipient host cells.

10

cDNA Library. By "cDNA library" is meant a collection of recombinant DNA molecules containing cDNA inserts which together comprise the entire genome of an organism. Such a cDNA library may be prepared by methods known to those of

15 skill, and described, for example, in Maniatis, *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 2d. ed. (1989). Generally, RNA is first isolated from the cells of an organism from whose genome it is desired to clone a

20 particular gene. Preferred for the purposes of the present invention are mammalian, and particularly human, cell lines.

Cloning Vehicle. A plasmid or phage DNA or other DNA sequence which is able to replicate in a host cell. The

25 cloning vehicle is characterized by one or more endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the DNA, which may contain a marker suitable for use in the identification of transformed cells.

30 Markers may include for example, tetracycline resistance or ampicillin resistance. The word "vector" can be used to connote a cloning vehicle.

Expression Control Sequence. An "expression control sequence" is a sequence of nucleotides that controls or

35 regulates expression of structural genes when operably

linked to those genes. These include, for example, the lac
systems, the trp system major operator and promoter regions
of the phage lambda, the control region of fd coat protein
and other sequences known to control the expression of genes
in prokaryotic or eukaryotic cells. Expression control
sequences will vary depending on whether the vector is
designed to express the operably linked gene in a
prokaryotic or eukaryotic host, and may contain
transcriptional elements such as enhancer elements,
termination sequences, tissue-specificity elements, and/or
translational initiation and termination sites.

Expression vehicle. An "expression vehicle" is a
vehicle or vector similar to a cloning vehicle but which is
capable of expressing a gene which has been cloned into it,
after transformation into a host. The cloned gene is
usually placed under the control of (i.e., operably linked
to) an expression control sequence.

Operator. A DNA sequence capable of interacting with
the specific repressor, thereby controlling the
transcription of adjacent gene(s).

Promoter. The term "promoter" is intended to refer to
a DNA sequence which can be recognized by an RNA polymerase.
The presence of such a sequence permits the RNA polymerase
to bind and initiate transcription of operably linked gene
sequences.

Promoter region. The term "promoter region" is
intended to include the promoter as well as other gene
sequences which may be necessary for the initiation of
transcription. The presence of a promoter region is,
therefrom sufficient to cause the expression of an operably
linked gene sequence.

Operably Linked. As used herein, the term "operably linked" means that the promoter controls the initiation of expression of the gene. A promoter is operably linked to a sequence of proximal DNA if upon introduction into a host cell the promoter determines the transcription of the proximal DNA sequence or sequences into one or more species of RNA. A promoter is operably linked to a DNA sequence if the promoter is capable of initiating transcription of that DNA sequence.

Prokaryote. The term "prokaryote" is meant to include all organisms without a true nucleus, including bacteria.

Eukaryote. The term "eukaryote" is meant to include organisms and cells which have a true nucleus, including mammalian cells.

Host. The term "host" is meant to include not only prokaryotes, but also eukaryotes such as yeast and filamentous fungi, as well as plant and animal cells. The term includes an organism or cell that is the recipient of a replicable expression vehicle.

Substantially homologous. The term "substantially homologous" as used herein refers to the ability of a first DNA sequence encoding Bcl-Y to hybridize to a second DNA sequence encoding the foregoing, under stringent conditions, for example, at about 0.1x sodium citrate sodium chloride buffer (SSC) at a temperature of about 65°C.

Substantially pure. The term "substantially pure" means that the protein or molecule of interest is essentially free from any other detectable biological constituents.

Functional Equivalent. As used herein, a "functional
equivalent" of the Bcl-Y protein is a protein which
possesses a biological activity or immunological
characteristic substantially similar to a biological
activity or immunological characteristic of non-recombinant
Bcl-Y. The term "functional equivalent is intended to
include the "fragments," "variants," "analogues,"
"homologues," or "chemical derivatives" of a molecule which
possess the biological activity of the Bcl-Y protein of the
invention.

Fragment. A "fragment" of a molecule such as Bcl-Y is
meant to refer to any variant of the molecule which possess
the biological activity of the Bcl-Y protein.

Variant. A "variant" of a molecule is meant to refer
to a molecule substantially similar in structure and
biological activity or immunological characteristics to
either the entire molecule, or to a fragment thereof. Thus,
provided that two molecules possess a similar activity, they
are considered variants as that term is used herein even if
the composition or secondary, tertiary, or quaternary
structure of one of the molecules is not identical to that
found in the other, or if the sequence of amino acid
residues is not identical.

Analog. An "analog" of a molecule is meant to refer to
a molecule substantially similar in function to either the
entire molecule or to a fragment thereof. As used herein, a
molecule is said to be a "chemical derivative" of another
molecule when it contains additional chemical moieties not
normally a part of the molecule. Such moieties may improve
the molecule's solubility, absorption, biological half life,
etc. The moieties may alternatively decrease the toxicity
of the molecule, eliminate or attenuate any undesirable side
effect of the molecule, etc. Moieties capable of mediating

such effects are described, for example, in Remington's
Pharmaceutical Sciences (1980). Procedures for coupling
such moieties to a molecule are well known in the art.

Anti-Antibody. By the term "anti-antibody" is intended
an antibody directed against antigenic determinants on
another antibody.

Anti-Idiotypic Antibody. By the term "anti-idiotypic
antibody" is intended an antibody directed against an
idiotypic determinant of another antibody.

Idiotope. By the term "idiotope" is intended an
idiotypic determinant, i.e., an antigenic determinant on a
variable domain of an immunoglobulin molecule.

Idiotype. By the term "idiotype" is intended a set of
one or more idiotopes that distinguish a clone of
immunoglobulin producing cells from other clones. Idiotypes
occur in the variable domains of immunoglobulin molecules
and may be within, near to, or outside of the antigen
binding site; antibodies to idiotypes located within or near
the antigen biding site will prevent the immunoglobulin from
combining with the antigen.

Idiotype-anti-idiotype Network. By the term "idiotype-
anti-idiotype network" is intended a B-cell regulatory
mechanism. Activation of a B cell results in a clone of
plasma cells producing immunoglobulin of a single idiotype,
which, because it was previously present in very small
quantities, can be recognized as "nonself" and results in
the production of anti-idiotypic antibodies directed against
its idiotypic determinants. There can also be anti-anti-
idiotypic antibodies directed against the second antibodies,
antibodies directed against them, and so forth. These
antibodies react with antigen receptors on B cells and T

helper and suppressor cells, as well as with circulating
antibodies, to enhance or suppress production of the initial
antibody by various mechanisms.

5   *Modulate*. By the term "modulate" is intended, for the
purposes of the present invention, the induction of
apoptosis by the administration of the Bcl-Y protein of the
invention, an active fragment thereof, a functional
equivalent thereof, and/or the suppression or induction of
10 apoptosis by the administration of a Bcl-Y hybrid or Bcl-Y
mutant, or the administration of a vector containing cDNA
encoding any of the foregoing, to the particular cells of an
individual suffering from any degenerative disorder which
results in inappropriate cell growth, for example, including
15 lymphomas, genotypic tumors, cancer, or, disorders
characterized by inappropriate cell death, for example,
including AIDS which results in T-cell death, in order to
stabilize inappropriate cell proliferation or inappropriate
cell death and preferably to restore normal cell behavior.

20

    Administration. By the term "administration" is
intended any mode of administration which results in the
delivery of the therapeutic agent across the cell membrane
and into the desired cell. The site of administration and
25 cells will be selected by one of ordinary skill in the art
based upon an understanding of the particular degenerative
disorder being treated. In addition, the dosage, dosage
frequency, and length of course of treatment, can be
determined and optimized by one of ordinary skill in the art
30 depending upon the particular degenerative disorder being
treated. The particular mode of administration can also be
readily selected by one of ordinary skill in the art and can
include, for example, oral, intravenous, subcutaneous,
intramuscular, etc., with the requirement that the
35 therapeutic agent cross the cell membrane. The therapeutic
agent of the present invention can be the Bcl-Y protein and/

or functional equivalents thereof and/or Bcl-Y hybrids or
Bcl-Y mutants and/or a vector containing cDNA encoding the
foregoing.

5   *Therapeutic Agent.*   By the term "therapeutic agent" is
intended the present Bcl-Y protein, fragments, functional
equivalents and/or hybrids or mutants thereof as well as
vectors containing cDNA encoding any of the foregoing. The
present therapeutic agent can be administered alone or in
10 combination with and/or concurrently with other suitable
drugs and/or courses of therapy.

        Degenerative Disorder.   By the term "degenerative
disorder" is intended for purposes of this invention, any
15 disorder characterized by inappropriate cell proliferation
or inappropriate cell death or in some cases, both. By the
term "inappropriate cell proliferation" is intended a
statistically significant increase in cell number as
compared to the proliferation of that particular cell type
20 in the normal population. Also included are disorders
whereby a cell is present and/or persists in an
inappropriate location, e.g., the presence of fibroblasts in
lung tissue after acute lung injury. For example, such
cells include cancer cells which exhibit the properties of
25 invasion and metastasis and are highly anaplastic. Such
cells include but are not limited to, cancer cells
including, for example, tumor cells. By the term
"inappropriate cell death" is intended a statistically
significant decrease in cell number as compared to the
30 presence of that particular cell type in the normal
population. Such underrepresentation may be due to a
particular degenerative disorder, including, for example,
AIDS (HIV), which results in the inappropriate death of
T-cells, autoimmune diseases which are characterized by
35 inappropriate cell death. By the term "autoimmune disease"
is intended a disorder caused by an immune response directed

against self antigens. Such diseases are characterized by the presence of circulating autoantibodies or cell-mediated immunity against autoantigens in conjunctions with inflammatory lesions caused by immunologically competent cells or immune complexes in tissues containing the autoantigens. Such diseases include systemic lupus, erythematosus (SLE), rheumatoid arthritis.

Suppression. By the term "suppression" is intended for the purposes of this invention the result achieved by administering an amount of a therapeutic agent containing Bcl-Y hybrids or Bcl-Y mutants thereof effective to suppress apoptosis in an individual suffering from a degenerative disorder characterized by inappropriate cell death. Suppression of apoptosis is achieved when the numbers of the particular affected cell type remain stable or increase in number to a level within the range observed in the normal cell population. By the term "stable" is intended the state achieved when a statistically significant decrease in cell number is no longer observed in the individual being treated, as compared to the cell number observed at the onset of the course of treatment.

Induction. By the term "induction" is intended for the purposes of this invention the result achieved by the administration of an amount of a therapeutic agent containing the Bcl-Y of the invention effective to induce apoptosis in cells of an individual suffering from a degenerative disorder characterized by inappropriate cell proliferation. The induction of apoptosis is achieved when cell numbers remain stable or decrease to a level within the range observed in the normal cell population. By the term "stable" is intended the state achieved during the course of treatment when a statistically significant increase in cell proliferation is no longer observed as compared to the cell number observed at the onset of the course of treatment.

One of ordinary skill in the art can readily determine whether the induction of apoptosis has been achieved.

Bcl-Y Hybrid.   By the term "Bcl-Y hybrid" is intended for the purposes of this invention, proteins which are hybrid proteins of the present Bcl-Y proteins, fragments thereof, and/or functional equivalents or mutants thereof, with other apoptosis associated proteins encoded by genes including, for example, Bcl-2, Bax, c-myc, LMW5-HL, Bcl-Y, $Bcl-X_L$, $Bcl-X_S$, BHRF-1, Mcl, A1 and ced9, fragments thereof and/or functional equivalents thereof, in order to produce a protein which exhibits enhanced, decreased, or intermediate apoptosis induction or suppression activity as compared to the activity of Bcl-Y alone.   Such hybrids can be produced, for example, by fusing the first half of the coding region of the Bcl-Y cDNA with the second half of the coding region of the cDNA for Bcl-2, or Bax, or $Bcl-X_L$, or $Bcl-X_S$ or vice versa.   Additionally, by adding or replacing segments of $Bcl-2$, Bax, $Bcl-X_L$ or $Bcl-X_S$ to the Bcl-Y cDNA, chimeric gene products of therapeutic value can be generated.   One of ordinary skill in the art can readily produce and employ such hybrids using techniques well known in the art.   One of ordinary skill in the art can readily determine whether a particular hybrid exhibits enhanced, decreased or intermediate apoptosis induction or suppression activity using known screening methods and as described herein.

Normal Cell Behavior.   By the term "normal cell behavior" is intended for the purposes of this invention, cells in which apoptosis proceeds normally.   Normal cell behavior is observed in an organism which is able to remove senescent, damaged, or abnormal cells that could interfere with organ function or develop into tumors.   Apoptosis which proceeds normally represents a coordinated cellular response to noxious stimuli that are not immediately lethal.

Patient/Individual. By the term "patient" or
"individual" is intended for the purposes of the present
invention, animals, including humans and mammals, who suffer
from a degenerative disorder.

5

Bcl-Y mutant. By the term "Bcl-Y mutant" is intended
for the purposes of the present invention a mutant of Bcl-Y
which exhibits the reverse (apoptosis suppression) activity
of the Bcl-Y protein of the invention due to the
10 substitution of one or more amino acids or corresponding
nucleotides.

Apoptosis associated protein Bcl-Y. By the term
"apoptosis associated protein Bcl-Y" is intended for the
15 purposes of the present invention both the isolated
naturally occurring and isolated recombinantly produced
protein (i.e., synthetic Bcl-Y) which exhibits, *inter alia*,
apoptosis induction from human tissue including, for
example, tumor cells and established human cell lines, and
20 from tissues of other animals including mammals. This term
includes any analog, homolog, mutant or derivative of
isolated naturally occurring Bcl-Y including fragments
having less than the naturally occurring number of amino
acids, such as partial fragments of natural or synthetic
25 Bcl-Y which retain the biological or immunological
characteristics of the polypeptide disclosed in this
application.
    This term also includes any peptide which contains the
sequence of an isolated naturally occurring Bcl-Y protein,
30 or analog or homolog thereof, together with one or more
flanking amino acids, which retains the biological or
immunological characteristics of the Bcl-Y protein of the
invention.
    The present invention pertains to both the expression
35 of full-length Bcl-Y and of functional derivatives of this
protein, including allelic variants of Bcl-Y and species or

viral homologs of Bcl-Y. Species homologs can be identified, isolated and recombinantly produced using the present nucleotide probes and procedures as described herein, being methods well known in the art. Further, one

5 of ordinary skill in the art can readily determine whether a particular peptide is a functional equivalent of Bcl-Y using methods well known in the art.

More specifically, this term includes proteins encoded by the nucleotide sequence as shown in Fig. 4, and proteins

10 having the amino acid sequence as shown in Fig. 4, allelic variants, species homologs and viral homologs thereof, as well as functional derivatives thereof including fragments which retain the biological characteristics of the present Bcl-Y, and proteins that are substantially homologous

15 thereto, which retain the biological or immunological characteristics of the Bcl-Y protein of the invention.

Also, this term includes mutant proteins which differ from the present Bcl-Y protein by one or more amino acids or corresponding nucleotides, which difference results in the

20 reversal of function of Bcl-Y, i.e., the mutant protein exhibits apoptosis suppression activity. Accordingly, the present Bcl-Y proteins and the mutant proteins, or their corresponding polynucleotide sequences, are useful for the regulation of apoptosis in cells.

25

II. **Genetic Engineering of Bcl-Y, Functional Equivalents
     Thereof and Mutants Thereof**

30   This invention comprises amino acid sequences of Bcl-Y or Bcl-Y mutant, genetic sequences coding for such amino acid sequences, expression vehicles containing the genetic sequences, hosts transformed therewith and recombinant Bcl-Y and antisense RNA produced by such transformed host

35 expression. The invention further comprises antibodies

directed against Bcl-Y and/or fragments thereof or against
Bcl-Y mutants.

    The process for genetically engineering such protein
sequences, according to the invention, is facilitated
through the cloning of genetic sequences which are capable
of encoding the peptide and through the expression of such
genetic sequences. As used herein, the term "genetic
sequences" is intended to refer to a nucleic acid molecule
(preferably DNA). Genetic sequences which are capable of
encoding the proteins are derived from a variety of sources.
These sources include genomic DNA, cDNA, synthetic DNA, and
combinations thereof. The preferred source of the genomic
DNA or mRNA is human tissue including heart, lung, tumor
cells, brain, placenta, liver, skeletal muscle, kidney and
pancreas. The mRNA may then be used to obtain cDNA by
techniques known to those skilled in the art. Probes may be
synthesized based on the nucleotide sequence of Bcl-Y by
methods known in the art.

    The Bcl-Y protein or fragment genomic DNA of the
invention may or may not include naturally occurring
introns. Moreover, such genomic DNA may be obtained in
association with the 5' promoter region of the Bcl-Y protein
gene sequences and/or with the 3' transcriptional
termination region. Further, such genomic DNA may be
obtained in association with the genetic sequences which
encode the 5' non-translated region of the Bcl-Y protein
mRNA and/or with the genetic sequences which encode the 3'
non-translated region. To the extent that a host cell can
recognize the transcriptional and/or translational
regulatory signals associated with the expression of the
mRNA and protein, the 5' and/or 3' non-transcribed regions
of the native gene, and/or the 5' and/or 3' non-translated
regions of the mRNA, may be retained and employed for
transcriptional and translational regulation. Bcl-Y protein
genomic DNA can be extracted and purified from human tissue
by means well known in the art (for example, see *Guide to*

Molecular Cloning Techniques, S.L. Berger, *et al.,* eds., Academic Press (1987)).

Alternatively, mRNA can be isolated from any cell which produces or expresses the protein, and used to produce cDNA by means well known in the art (for example, see *Guide to Molecular Cloning Techniques,* S.L. Berger, *et al.,* eds., *supra.* Preferably, the mRNA preparation used will be enriched in mRNA coding for such Bcl-Y protein, either naturally, by isolation from cells which are producing large amounts of the protein, or *in vitro,* by techniques commonly used to enrich mRNA preparations of specific sequences, including for example sucrose gradient centrifugation, or PCR. cDNA can then be prepared for example, by reverse transcription. The cDNA can then be amplified by PCR using suitable primers.

For cloning into a vector, such suitable DNA preparations (either human genomic DNA or cDNA) are randomly sheared or enzymatically cleaved, respectively, and ligated into appropriate vectors to form a recombinant gene (either genomic or cDNA) library. A DNA sequence encoding the Bcl-Y protein or its functional equivalents may be inserted into a DNA vector in accordance with conventional techniques, including blunt-ending or staggered-ending termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases. Techniques for such manipulations are disclosed, for example, by Sambrook, *et al.,* (*In: Molecular Cloning, A Laboratory Manual,* Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 2d. ed. (1989)), and are well known in the art.

Libraries containing the Bcl-Y protein clones may be screened and a Bcl-Y clone identified by any means which specifically selects for Bcl-Y protein DNA such as, for example, (a) by hybridization with an appropriate nucleic

acid probe(s) containing a sequence specific for the DNA of this protein, or (b) by hybridization-selected translational analysis in which native mRNA which hybridizes to the clone in question is translated *in vitro* and the translation
5   products are further characterized, or, (c) if the cloned genetic sequences are themselves capable of expressing mRNA, by immunoprecipitation of a translated Bcl-Y or fragment product produced by the host containing the clone.

        Oligonucleotide probes specific for the protein which
10 can be used to identify clones to this protein can be designed from knowledge of the amino acid sequence of the Bcl-Y protein. The sequence of amino acid residues in a peptide is designated herein either through the use of their commonly employed three-letter designations or by their
15 single-letter designations. A listing of these three-letter and one-letter designations may be found in textbooks such as *Biochemistry*, 2ed., Lehninger, A., Worth Publishers, New York, NY (1975). When the amino acid sequence is listed horizontally, the amino terminus is intended to be on the
20 left end whereas the carboxy terminus is intended to be at the right end. The residues of amino acids in a peptide may be separated by hyphens. Such hyphens are intended solely to facilitate the presentation of a sequence.

        Because the genetic code is degenerate, more than one
25 codon may be used to encode a particular amino acid (Watson, J.D., *In: Molecular Biology of the Gene*, 3rd Ed., W.A. Benjamin, Inc., Menlo Park, CA (1977), pp. 356-357). The peptide fragments are analyzed to identify sequences of amino acids which may be encoded by oligonucleotides having
30 the lowest degree of degeneracy. This is preferably accomplished by identifying sequences that contain amino acids which are encoded by only a single codon.

        Although occasionally an amino acid sequence may be encoded by only a single oligonucleotide sequence,
35 frequently the amino acid sequence may be encoded by any of a set of similar oligonucleotides. Importantly, whereas all

of the members of this set contain oligonucleotide sequences which are capable of encoding the same peptide fragment and, thus, potentially contain the same oligonucleotide sequence as the gene which encodes the peptide fragment, only one member of the set contains the nucleotide sequence that is identical to the exon coding sequence of the gene. Because this member is present within the set, and is capable of hybridizing to DNA even in the presence of the other members of the set, it is possible to employ the unfractionated set of oligonucleotides in the same manner in which one would employ a single oligonucleotide to clone the gene that encodes the peptide.

Using the genetic code (Watson, J.D., In: Molecular Biology of the Gene, 3rd Ed., W.A. Benjamin, Inc., Menlo Park, CA (1977)), one or more different oligonucleotides can be identified from the amino acid sequence, each of which would be capable of encoding the present Bcl-Y or fragment protein. The probability that a particular oligonucleotide will, in fact, constitute the actual protein coding sequence can be estimated by considering abnormal base pairing relationships and the frequency with which a particular codon is actually used (to encode a particular amino acid) in eukaryotic cells. Such "codon usage rules" are disclosed by Lathe, et al., J. Molec. Biol. 183:1-12 (1985). Using the "codon usage rules" of Lathe, a single oligonucleotide sequence, or a set of oligonucleotide sequences, that contains a theoretical "most probable" nucleotide sequence capable of encoding the Bcl-Y protein sequences is identified.

The suitable oligonucleotide, or set of oligonucleotides, which is capable of encoding a fragment of the Bcl-Y protein gene (or which is complementary to such an oligonucleotide, or set of oligonucleotides) may be synthesized by means well known in the art (see, for example, Synthesis and Application of DNA and RNA, S.A. Narang, ed., 1987, Academic Press, San Diego, CA) and

employed as a probe to identify and isolate the cloned Bcl-Y
protein gene by techniques known in the art. Techniques of
nucleic acid hybridization and clone identification are
disclosed by Maniatis, *et al.* (*In: Molecular Cloning, A*
5 *Laboratory Manual*, Cold Spring Harbor Laboratories, Cold
Spring Harbor, NY (1982)); Berger, *et al.*, (*In: Guide to*
Molecular Cloning Techniques, Academic Press (1988));
Sambrook, *et al.*, (*In: Molecular Cloning, A Laboratory*
Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY, 2d
10 ed. (1989); and by Hames, *et al.* (*In: Nucleic Acid*
Hybridization, A Practical Approach, IRL Press, Washington,
DC (1985)), which references are herein incorporated by
reference. Those members of the above-described gene
library which are found to be capable of such hybridization
15 are then analyzed to determine the extent and nature of the
Bcl-Y protein encoding sequences which they contain.

To facilitate the detection of the desired Bcl-Y or
fragment protein DNA encoding sequence, the above-described
DNA probe is labeled with a detectable group or label. Such
20 detectable group or label can be any material having a
detectable physical or chemical property. Such materials
have been well-developed in the field of nucleic acid
hybridization and in general most any label useful in such
methods can be applied to the present invention.
25 Particularly useful are radioactive labels, such as ^{32}P, ^{3}H,
^{14}C, ^{35}S, ^{125}I, or the like. Any radioactive label may be
employed which provides for an adequate signal and has a
sufficient half-life. The oligonucleotide may be
radioactively labeled, for example, by "nick-translation" by
30 well-known means, as described in, for example, Rigby, *et*
al., *J. Mol. Biol. 113*:237 (1977) and by T4 DNA polymerase
replacement synthesis as described in, for example, Deen, *et*
al., *Anal. Biochem. 135*:456 (1983).

Alternatively, polynucleotides are also useful as
35 nucleic acid hybridization probes when labeled with a
non-radioactive marker such as biotin, an enzyme or a

fluorescent or chemiluminescent group. See, for example,
Leary, et al., Proc. Natl. Acad. Sci, USA 80:4045 (1983);
Renz, et al., Nucl. Acids Res. 12:3435 (1984); and Renz, M.,
EMBO J. 6:817 (1983).

5   Thus, the actual identification of the Bcl-Y protein
sequences permits the identification of a theoretical "most
probable" DNA sequence, or a set of such sequences, capable
of encoding such a peptide. By constructing an
oligonucleotide complementary to this theoretical sequence
10 (or by constructing a set of oligonucleotides complementary
to the set of "most probable" oligonucleotides), one obtains
a DNA molecule (or set of DNA molecules), capable of
functioning as a probe(s) for the identification and
isolation of clones containing the Bcl-Y protein gene.

15   In an alternative way of cloning the Bcl-Y protein
gene, a library is prepared using an expression vector, by
cloning DNA or, more preferably, cDNA prepared from a cell
capable of expressing the Bcl-Y protein, into an expression
vector. The library is then screened for members which
20 express the Bcl-Y protein, for example, by screening the
library with antibodies to the Bcl-Y protein.

        The above discussed methods are, therefore, capable of
identifying genetic sequences which are capable of encoding
Bcl-Y proteins or fragments thereof. In order to further
25 characterize such genetic sequences, and, in order to
produce the recombinant protein, it is desirable to express
the proteins which these sequences encode. Such expression
identifies those clones which express proteins possessing
characteristics of the Bcl-Y proteins. Such characteristics
30 may include the ability to specifically bind antibody to the
Bcl-Y protein and the ability to elicit the production of an
antibody or antibodies which are capable of binding to the
Bcl-Y protein.

35

III. Expression of Bcl-Y protein, Fragments Thereof, Functional Equivalents Thereof, and Mutants Thereof

To express the Bcl-Y protein or a functional
equivalent, or mutant thereof, transcriptional and
translational signals recognizable by an appropriate host
are necessary. The cloned Bcl-Y encoding sequences,
obtained, for example, through the methods described above,
and preferably in a double-stranded form, may be operably
linked to sequences controlling transcriptional expression
in an expression vector, and introduced into a host cell,
either prokaryotic or eukaryotic, to produce recombinant
Bcl-Y protein or a functional equivalent thereof. Depending
upon which strand of the Bcl-Y encoding sequence is operably
linked to the sequences controlling transcriptional
expression, it is also possible to express Bcl-Y antisense
RNA or a functional equivalent thereof.

Expression of Bcl-Y in different hosts may result in
different post-translational modifications which may alter
the properties of the Bcl-Y. The present invention
encompasses the expression of the Bcl-Y protein, or
functional equivalent thereof, or Bcl-Y mutant, in
prokaryotic or eukaryotic cells, and particularly,
eukaryotic expression is preferred.

Preferred prokaryotic hosts include bacteria such as *E.
coli*, *Bacillus*, *Streptomyces*, *Pseudomonas*, *Salmonella*,
Serratia, etc. The most preferred prokaryotic host is *E.
coli*. Other enterobacteria such as *Salmonella typhimurium*
or *Serratia marcescens*, and various *Pseudomonas* species may
also be utilized. Under such conditions, the protein may
not be glycosylated. The procaryotic host must be
compatible with the replicon and control sequences in the
expression plasmid.

To express the Bcl-Y protein (or a functional
equivalent thereof) or Bcl-Y mutant in a prokaryotic cell
(such as, for example, *E. coli*, *B. subtilis*, *Pseudomonas*,

Streptomyces, etc.), it is necessary to operably link the Bcl-Y encoding sequence to a functional prokaryotic promoter. Such promoters may be either constitutive or, more preferably, regulatable (i.e., inducible or

5 derepressible). Examples of constitutive promoters include the *int* promoter of bacteriophage lambda, the *bla* promoter of the Beta-lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene of pBR325, etc. Examples of inducible prokaryotic promoters include

10 the major right and left promoters of bacteriophage lambda (P_L and P_R), the *trp, recA, lacZ, lacI,* and *gal* promoters of *E. coli,* the alpha-amylase (Ulmanen, I., *et al., J. Bacteriol. 162:*176-182 (1985)) and the sigma-28-specific promoters of *B. subtilis* (Gilman, M.Z., *et al., Gene 32:*11-

15 20 (1984)), the promoters of the bacteriophages of *Bacillus* (Gryczan, T.J., *In: The Molecular Biology of the Bacilli,* Academic Press, Inc., NY (1982)), and *Streptomyces* promoters (Ward, J.M., *et al., Mol. Gen. Genet. 203:*468-478 (1986)). Prokaryotic promoters are reviewed by Glick, B.R., (*J. Ind.*

20 *Microbiol. 1:*277-282 (1987)); Cenatiempo, Y. (*Biochimie 68:*505-516 (1986)); and Gottesman, S. (*Ann. Rev. Genet. 18:*415-442 (1984)).

Proper expression in a prokaryotic cell also requires the presence of a ribosome binding site upstream of the

25 gene-encoding sequence. Such ribosome binding sites are disclosed, for example, by Gold, L., *et al.* (*Ann. Rev. Microbiol. 35:*365-404 (1981)).

Especially preferred eukaryotic hosts include mammalian cells either *in vivo,* in animals or in tissue culture.

30 Expression of the Bcl-Y in eukaryotic hosts requires the use of regulatory regions functional in such hosts, and preferably eukaryotic regulatory systems. A wide variety of transcriptional and translational regulatory sequences can be employed, depending upon the nature of the eukaryotic

35 host. The transcriptional and translational regulatory signals can also be derived from the genomic sequences of

viruses which infect eukaryotic cells, such as adenovirus,
bovine papilloma virus, Simian virus, herpes virus, or the
like. Preferably, these regulatory signals are associated
with a particular gene which is capable of a high level of
5 expression in the host cell.

In eukaryotes, where transcription is not linked to
translation, such control regions may or may not provide an
initiator methionine (AUG) codon, depending on whether the
cloned sequence contains such a methionine. Such regions
10 will, in general, include a promoter region sufficient to
direct the initiation of RNA synthesis in the host cell.
Promoters from heterologous mammalian genes which encode
mRNA product capable of translation are preferred, and
especially, strong promoters such as the promoter for actin,
15 collagen, myosin, etc., can be employed provided they also
function as promoters in the host cell. Preferred
eukaryotic promoters include the promoter of the mouse
metallothionein I gene (Hamer, *et al.*, *J. Mol. Appl. Gen.*
1:273-288 (1982)); the TK promoter of Herpes virus
20 (McKnight, S., *Cell 31*:355-365 (1982)); the SV40 early
promoter (Benoist, *et al.* *Nature (London) 290*:304-310
(1981)); in yeast, the yeast *gal4* gene promoter (Johnston,
et al. Proc. Natl. Acad. Sci. USA 79:6971-6975 (1982);
Silver, *et al.*, *Proc. Natl. Acad. Sci. USA 81*:5951-5955
25 (1984)) or a glycolytic gene promoter may be used.

As is widely known, translation of eukaryotic mRNA is
initiated at the codon which encodes the first methionine.
For this reason, it is preferable to ensure that the linkage
between a eukaryotic promoter and a DNA sequence which
30 encodes the Bcl-Y protein, or a functional equivalent
thereof, does not contain any intervening codons which are
capable of encoding a methionine. The presence of such
codons results either in the formation of a fusion protein
(if the AUG codon is in the same reading frame as the Bcl-Y
35 encoding DNA sequence) or a frame-shift mutation (if the AUG

codon is not in the same reading frame as the Bcl-Y encoding sequence).

If desired, a fusion product of the Bcl-Y may be constructed. For example, the sequence coding for the Bcl-Y or fragment thereof may be liked to a signal sequence which will allow secretion of the protein from or the compartmentalization of the protein in, a particular host. Such signal sequences may be designed with or without specific protease sites such that the signal peptide sequence is amenable to subsequent removal.

Transcriptional initiation regulatory signals can be selected which allow for repression or activation, so that expression of the operably linked genes can be modulated. Of interest are regulatory signals which are temperature-sensitive, such that by varying the temperature, expression can be repressed or initiated, or which are subject to chemical regulation, e.g., by a metabolite. Also of interest are constructs wherein the Bcl-Y mRNA and antisense RNA are provided in a transcribable form, but with different promoters or other transcriptional regulatory elements such that induction of Bcl-Y mRNA expression is accompanied by repression of antisense RNA expression, and/ or repression of Bcl-Y mRNA expression is accompanied by induction of antisense RNA expression.

Translational signals are not necessary when it is desired to express Bcl-Y antisense RNA sequences.

If desired, the non-transcribed and/or non-translated regions 3' to the sequence coding for the Bcl-Y protein can be obtained by the above-described cloning methods. The 3'-non-transcribed region may be retained for its transcriptional termination regulatory sequence elements; the 3'-non-translated region may be retained for its translation termination regulatory sequence elements, or for those elements which direct polyadenylation in eukaryotic cells. Where the native expression control sequence signals

do not function satisfactorily in the host cell, then
sequences functional in the host cell may be substituted.

The vectors of the invention may further comprise other
operably linked regulatory elements such as enhancer
sequences, or DNA elements which confer tissue or cell-type
specific expression on an operably linked gene.

To transform a mammalian cell with the DNA constructs
of the invention many vector systems are available,
depending upon whether it is desired to insert the Bcl-Y DNA
construct into the host cell chromosomal DNA, or to allow it
to exist in an extrachromosomal form.

If the Bcl-Y DNA encoding sequence and an operably
linked promoter are introduced into a recipient eukaryotic
cell as a non-replicating DNA (or RNA) molecule, which may
either be a linear molecule or a closed covalent circular
molecule which is incapable of autonomous replication, then
the expression of the Bcl-Y protein may occur through the
transient expression of the introduced sequence.

Genetically stable transformants may be constructed
with vector systems, or transformation systems, whereby
Bcl-Y DNA is integrated into the host chromosome. Such
integration may occur *de novo* within the cell or, in a
preferred embodiment, be assisted by transformation with a
vector which functionally inserts itself into the host
chromosome, for example, with retroviral vectors,
transposons or other DNA elements which promote integration
of DNA sequences into chromosomes. A vector is employed
which is capable of integrating the desired gene sequences
into a mammalian host cell chromosome.

Cells which have stably integrated the introduced DNA
into their chromosomes are selected by also introducing one
or more markers which allow for selection of host cells
which contain the expression vector in the chromosome, for
example, the marker may provide biocide resistance, e.g.,
resistance to antibiotics, or heavy metals, such as copper,
or the like. The selectable marker gene can either be

directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection.

In another embodiment, the introduced sequence is incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors may be employed for this purpose, as outlined below.

Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

Preferred eukaryotic plasmids include those derived from the bovine papilloma virus, vaccinia virus, SV40, and, in yeast, plasmids containing the 2-micron circle, etc., or their derivatives. Such plasmids are well known in the art (Botstein, *et al., Miami Wntr. Symp. 19*:265-274 (1982); Broach, J.R., *In: The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 445-470 (1981); Broach, J.R., *Cell 28*:203-204 (1982); Bollon, *et al., J. Clin. Hematol. Oncol. 10*:39-48 (1980); Maniatis, T., *In: Cell Biology: A Comprehensive Treatise,* Vol. 3, "Gene Expression," Academic Pres, NY, pp. 563-608 (1980)), and are commercially available. For example, mammalian expression vector systems which utilize the MSV-LTR promoter to drive expression of the cloned gene, and in which it is possible to contransfect with a helper virus to amplify plasmid copy number, and integrate the plasmid into the chromosomes of host cells, have been described (Perkins, *et al., Mol. Cell Biol. 3*:1123 (1983); Clontech, Palo Alto, California).

Once the vector or DNA sequence containing the construct(s) is prepared for expression, the DNA

construct(s) is introduced into an appropriate host cell by
any of a variety of suitable means, including transfection.
After the introduction of the vector, recipient cells are
grown in a selective medium, which selects for the growth of
5   vector-containing cells. Expression of the cloned gene
sequence(s) results in the production of the Bcl-Y protein,
or in the production of a fragment of this protein. This
expression can take place in a continuous manner in the
transformed cells, or in a controlled manner, for example,
10 expression which follows induction of differentiation of the
transformed cells (for example, by administration of
bromodeoxyuracil to neuroblastoma cells or the like).

The expressed protein is isolated and purified in
accordance with conventional conditions, such as extraction,
15 precipitation, chromatography, affinity chromatography,
electrophoresis, or the like.

Bcl-Y can be purified by growing the transformed host
cells under suitable conditions which are well known in the
art, the cells can be harvested and disrupted to extract
20 total cellular protein. The protein can then, for example,
be placed on a sizing column such as sepharose or agarose
beads, and proteins of the correct molecular weight can be
collected. The predicted molecular weight of Bcl-Y is 23.4
kD and it runs with an apparent molecular weight of
25 approximately 26 kD on SDS polyacrylamide gels.

Further purification can be effected by use of an
anti-Bcl-Y antibody. Such an antibody can be used to
immunoprecipitate Bcl-Y proteins from the set of cellular
proteins of the correct approximate molecular weight. Such
30 antibodies can, for example, be raised against polypeptides
synthesized according to the sequence or subsequences of the
sequence shown in Fig.4. Alternatively, the antibodies can
be raised against fusion proteins, which contain Bcl-Y
sequences as well as those of other proteins. After
35 immunoprecipitation, the Bcl-Y proteins can be released from

the antibodies to provide a substantially pure preparation
of Bcl-Y protein.

The Bcl-Y DNA coding sequences, of the present
invention may be used to obtain Bcl-Y antisense RNA genetic
sequences, inasmuch as the antisense RNA sequence will be
that sequence found on the opposite strand of the strand
transcribing the peptide core's mRNA. The antisense DNA
strand may also be operably linked to a promoter in an
expression vector such that transformation with this vector
results in a host capable of expression of a Bcl-Y antisense
RNA in the transformed cell. Antisense RNA and its
expression may be used to interact with an endogenous Bcl-Y
DNA or RNA in a manner which inhibits or represses
transcription or translation of the Bcl-Y genes in a highly
specific manner. Use of antisense RNA probes to block gene
expression is described, for example, in Lichtenstein, C.,
Nature 333:801-802 (1988).

**IV. Construction and Identification of Antibodies Raised
Against Bcl-Y, Functional Equivalents, Fragments,
Hybrids, or Mutants Thereof**

In the following description, reference will be made to
various methodologies well-known to those skilled in the art
of immunology. Standard reference works setting forth the
general principles of immunology include the work of Catty,
D., (*Antibodies, A Practical Approach*, Vol. 1, IRL Press,
Washington, DC (1988)); Klein, J., (*Immunology: The Science
of Cell-Noncell Discrimination*, John Wiley & Sons, New York
(1982)); Kennett, et al., (*Monoclonal Antibodies, Hybridoma:
A New Dimension in Biological Analyses*, Plenum Press, New
York (1980)); Campbell, A. ("Monoclonal Antibody
Technology," *In: Laboratory Techniques in Biochemistry and
Molecular Biology*, Vol. 13 (Burdon, R., et al., eds.),
Elsevier, Amsterdam (1984)); and Eisen, H.N. (*In:*

Microbiology, 3rd ed. (Davis, B.D., *et al.*, Harper & Row, Philadelphia (1980)).

An antibody is said to be "capable of binding" a molecule if it is capable of specifically reacting with the
5   molecule to thereby bind the molecule to the antibody. The term "epitope" is meant to refer to that portion of a hapten which can be recognized and bound by an antibody. An antigen may have one, or more than one epitope. An "antigen" is capable of inducing an animal to produce
10   antibody capable of binding to an epitope of that antigen. The specific reaction referred to above is meant to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which may be evoked by other antigens.
15   The term "antibody" (Ab) or "monoclonal antibody" (Mab) as used herein is meant to include intact molecules as well as fragments thereof (such as, for example, Fab and $F(ab)_2$ fragments) which are capable of binding an antigen. Fab and $F(ab)_2$ fragments lack the Fc fragment of intact antibody,
20   clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl, *et al.*, *J. Nucl. Med.* *24*:16-325 (1983)).

The antibodies of the present invention have specificity to one or more epitopes present on the Bcl-Y
25   peptide, or an idiotype on the present Bcl-Y. The antibodies of the invention can be polyclonal or monoclonal, provided that they are made with the present Bcl-Y polypeptide or fragment thereof as the immunogen. Both of these types of antibodies can be utilized in the
30   applications described herein.

The present antibodies can be used to detect the presence of the present Bcl-Y protein in a human tissue sample. The present Bcl-Y protein can be detected by contacting the sample with an imaging-effective amount of
35   the present detectably labeled appropriate antibody and detecting the label, thereby establishing the presence of

the Bcl-Y protein in the sample. Detection can be carried out by imaging *in vivo*. The Bcl-Y protein can also be detected by known immunoassay techniques, including, for example, RIA, ELISA, etc., using appropriate antibodies
5 according to the invention.

The antibodies of the present invention are prepared by any of a variety of known methods. For example, cells expressing the Bcl-Y protein can be administered to an animal in order to induce the production of serum containing
10 polyclonal antibodies that are capable of binding the Bcl-Y protein. For example, the Bcl-Y protein or fragment thereof is chemically synthesized and purified by HPLC to render it substantially free of contaminants. Such a preparation is then introduced into an animal in order to produce
15 polyclonal antisera of high specific activity.

Polyclonal antibodies can be generated in any suitable animal including, for example, mice, rabbits or goats. The Bcl-Y immunogenic peptide or fragment thereof can be injected by itself or linked to appropriate immunoactivating
20 carriers, such as Keyhole's limpet hemocyanin (KLH). See *Antibodies, A Practical Handbook*, Vols. I and II, D. Catty, ed., IRL Press, Washington, DC (1988).

Monoclonal antibodies can be prepared in various ways using techniques well understood by those having ordinary
25 skill in the art. For example, monoclonal antibodies can be prepared using hybridoma technology (Kohler, *et al.*, *Nature 256*:495 (1975); Kohler, *et al.*, *Eur. J. Immunol. 6*:511 (1976); Kohler, *et al.*, *Eur. J. Immunol. 6*:292 (1976); Hammerling, *et al.*, *In: Monoclonal Antibodies and T-Cell*
30 *Hybridomas*, Elsevier, N.Y., pp. 563-681 (1981)); *Monoclonal Antibodies - Hybridomas: A New Dimension in Biological Analysis*, edited by Roger H. Kennett, *et al.*, published by Plenum Press (1980). In general, such procedures involve immunizing an animal with the present Bcl-Y protein, or a
35 fragment thereof. The splenocytes of such animals are extracted and fused with a suitable myeloma cell line. Any

suitable myeloma cell line may be employed in accordance
with the present invention. After fusion, the resulting
hybridoma cells are selectively maintained in HAT medium,
and then cloned by limiting dilution as described by Wands,
5 et al., *Gastroenterology 80*:225-232 (1981). The hybridoma
cells obtained through such a selection are then assayed to
identify clones which secrete antibodies capable of binding
the Bcl-Y protein.

Through application of the above-described methods,
10 additional cell lines capable of producing antibodies which
recognize epitopes of the present Bcl-Y protein can be
obtained.

For example, additional hybridomas which produce
monoclonal antibodies which enable the detection of the
15 present Bcl-Y protein can be easily produced and isolated
with minimal screening. Hybridomas producing monoclonal
antibodies specific for epitopes which are found on the
present Bcl-Y protein are most effectively produced by first
immunizing an animal from which hybridomas can be produced
20 such as, for example, a Balb/c mouse, with initial
subcutaneous injections of Freund's adjuvant, followed by
booster injections within a few days. The fusion can be
carried out using any of the techniques commonly known to
those of ordinary skill in the art. The screening of the
25 hybridomas to determine which ones are producing monoclonal
antibodies specific for the present peptide is
straightforward and can be accomplished in a standard ELISA
or RIA format. For example, in an RIA screening format the
culture supernatant, or ascites fluid from a hybridoma
30 producing monoclonal antibody is reacted with ^{125}I-peptide.
The isolation of other hybridomas secreting mAbs of the same
specificity as those described herein can be accomplished by
the technique of anti-idiotypic screening. Potocmjak, *et
al., Science 215*:1637 (1982). Briefly, an anti-idiotypic
35 (anti-Id) antibody is an antibody which recognizes unique
determinants generally associated with the antigen-binding

site of an antibody. An Id antibody can be prepared by immunizing an animal of the same species and genetic type (e.g., mouse strain) as the source of the mAb with the mAb raised against the present Bcl-Y protein or fragment thereof
5 to which an anti-Id is being prepared. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody by producing an antibody to these idiotypic determinants (the anti-Id antibody).

By using an anti-Id antibody which is specific for
10 idiotypic determinants on a given mAb, it is then possible to identify other B cell or hybridoma clones sharing that idiotype. Idiotypic identity between the antibody product of two clones makes it highly probable that the antibody products of the two clones recognize the same antigenic
15 epitopes.

The anti-Id antibody may also be used as an "immunogen" to induce an immune response in yet another animal, producing a so-called anti-anti-Id antibody. The anti-anti-Id may be epitopically identical to the original
20 mAb which induced the anti-Id.

Thus, by using antibodies to the idiotypic determinants of a mAb, it is possible to identify other clones expressing antibodies of identical specificity.

Accordingly, mAbs generated against the present Bcl-Y
25 protein may be used to induce anti-Id antibodies in suitable animals, such as BALB/c mice. Spleen cells from such immunized mice are used to produce anti-Id hybridomas secreting anti-Id mAbs. Further, the anti-Id mAbs can be coupled to a carrier such as keyhole limpet hemocyanin (KLH)
30 and used to immunize additional BALB/c mice. Sera from these mice will contain anti-anti-Id antibodies that have the binding properties of the original mAb specific for the antigen epitope. The anti-Id mAbs thus have their own idiotypic epitopes, or "idiotopes" structurally similar to
35 the epitope being evaluated.

For replication, the hybridoma cells of this invention may be cultivated *in vitro* or *in vivo*. Production of high titers of mAbs *in vivo* production makes this the presently preferred method of production. Briefly, cells from the individual hybridomas are injected intraperitoneally into pristane-primed BALB/c mice to produce ascites fluid containing high concentrations of the desired mAbs. MAbs of isotype IgM or IgG may be purified from such ascites fluids, or from culture supernatants, using column chromatography methods well known to those of skill in the art.

Of special interest to the present invention are antibodies which are produced in humans, or are "humanized" (i.e., non-immunogenic in a human) by recombinant or other technology such that they will not be antigenic in humans, or will be maintained in the circulating serum of a recipient for a longer period of time.

Humanized antibodies may be produced, for example by replacing an immunogenic portion of an antibody with a corresponding, but non-immunogenic portion (i.e., chimeric antibodies) (Robinson, *et al.*, International Patent Publication PCT/US86/02269; Akira, *et al.*, European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison, *et al.*, European Patent Application 173,494; Neuberger, *et al.*, PCT Application WO 86/01533, Cabilly, *et al.*, European Patent Application 125,023; Better, *et al.*, *Science 240*:1041-1043 (1988); Liu, *et al.*, *Proc. Natl. Acad. Sci. USA 84*:3439-3443 (1987); Liu, *et al.*, *J. Immunol. 139*:3521-3526 (1987); Sun, *et al.*, *Proc. Natl. Acad. Sci. USA 84*:214-218 (1987); Nishimura, *et al.*, *Canc. Res. 47*:999-1005 (1987); Wood, *et al.*, *Nature 314*:446-449 (1985)); Shaw, *et al.*, *J. Natl. Cancer Inst. 80*:1553-1559 (1988). General reviews of "humanized" chimeric antibodies are provided by Morrison, S.L. (*Science, 229*:1202-1207 (1985)) and by Oi, *et al.*, *BioTechniques 4*:214 (1986)).

Suitable "humanized" antibodies can be alternatively produced as described by Jones, *et al.*, *Nature 321*:552-525 (1986); Verhoeyan, *et al.*, *Science 234*:1534 (1988), and Beidler, *et al.*, *J. Immunol.* 141:4053-4060 (1988).

The present Bcl-Y protein, fragments thereof, hybrids thereof, Bcl-Y mutants, or antibodies thereto can be utilized in immunoassays for the detection of the Bcl-Y protein in a human tissue sample. For example, antibodies against the present Bcl-Y protein can be used to detect the present Bcl-Y protein in a human tissue sample. The immunoassays can be competitive or sandwich, as is otherwise well known and they all depend on the formation of antibody-antigen immune complex. These assays are well known to those of skill in the art.

For purposes of the assays, the antibody or antigen can be immobilized or labeled. There are many carriers to which the antibody/antigen can be bound for immobilization and which can be used in the present invention. Well-known carriers include but are not limited to glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for purposes of the invention. Those skilled in the art will know many other suitable carriers for binding the antibody or antigen, or will be able to ascertain such, using routine experimentation.

Depending on the particular embodiment of the invention, one or more of the antibodies or antigen(s) peptide(s) will be coupled with a detectable label such as an enzyme, radioactive isotope, fluorescent compound, chemiluminescent compound, or bioluminescent compound.

Those of ordinary skill in the art will know of other suitable labels for binding to the antibodies or antigen(s) peptide(s) or will be able to ascertain such using routine experimentation. Furthermore, the binding of these labels

to the antibodies or antigen(s) can be done using standard techniques
commonly known to those of ordinary skill in the art.

The antibodies or antigen peptide(s) can be bound to an enzyme. This enzyme, in turn, when later exposed to its substrate will react with the substrate in such a manner as to produce a chemical moiety which can be detected, as, for example, by spectrophotometric or fluorometric means. Examples of enzymes that can be used to detectably label are amylate dehydrogenase, staphylococcal nuclease, delta-5-steroidisomerase, yeast alcoholdehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase, and acetylcholinesterase.

The presence of an antibody or antigen can also be detected by labeling the antibody or antigen with a radioactive isotope. The presence of the radioactive isotope can be determined by such means as the use of a gamma counter or a scintillation counter. Isotopes which are particularly useful are ^{3}H, ^{125}I, ^{32}P, ^{35}S, ^{14}C, ^{51}Cr, ^{36}Cl, ^{57}Co, ^{59}Fe, ^{75}Se, and ^{152}Eu.

It is possible to detect the presence of the antibody or antigen by labeling the antibody or antigen peptide with a fluorescent compound. When the fluorescently labeled antibody or antigen peptide is exposed to light of the proper wavelength, its presence can then be detected due to fluorescence of the dye. Among the most common fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde, and fluorescamine.

Another way in which the antibody or antigen can be detectably labeled is by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody or antigen peptide is then determined by detecting

the presence of luminescence that arises during the course
of a chemical reaction. Examples of particularly useful
chemiluminescent labeling compounds are luminol, isoluminol,
aromatic-acridinium ester, imidaxole, acridinium salt, and
5 oxalate ester.

Likewise, a bioluminescent compound may also be used to
label the antibody or antigen peptide. Bioluminescence is a
special type of chemiluminescence which is found in
biological systems and in which a catalytic protein
10 increases the efficiency of the chemiluminescent reaction.
The presence of a bioluminescent binding partner would be
determined by detecting the presence of luminescence.
Important bioluminescent compounds for purposes of labeling
are luciferin, luciferase, and aequorin.

15 The antibodies or antigen peptide(s) for use in the
assay of the invention are ideally suited for the
preparation of a kit. Such a kit may comprise a carrier
means being compartmentalized to receive in close
confinement one or more container means such as vials,
20 tubes, and the like, each of said container means comprising
one of the separate elements to be used in the method.

For example, one of the container means may comprise a
first antibody bound to an insoluble or partly soluble
carrier. A second container may comprise soluble,
25 detectably-labeled second antibody, in lyophilized form or
in solution. The carrier means may also contain a third
container means comprising a detectably labeled third
antibody in lyophilized form or in solution. Such a kit can
be used for sandwich assays.

30 In addition, the carrier means may also contain a
plurality of containers each of which comprises different,
predetermined amounts of the present Bcl-Y peptide. These
latter containers can then be used to prepare a standard
curve into which can be used to interpolate the results
35 obtained from the sample containing the unknown amount of
the present Bcl-Y protein.

Imaging can be carried out *in vitro* or *in vivo*. *In vitro* imaging can be done with the labels mentioned previously. *In vivo* imaging is done with diagnostically effective labeled antibodies. The term "diagnostically effective" means that the amount of detectably labeled antibody administered is sufficient to enable detection of the site of Bcl-Y protein presence when compared to a background signal.

Generally, the dosage of detectably-labeled antibody or antigen(s) for diagnosis will vary depending on considerations such as age, condition, sex, and extent of disease in the patient, counterindications, if any, and other variables, to be adjusted by the individual physician. Dosage can very from 0.01 mg/kg to 2,000 mg/kg, preferably 0.1 mg/kg to 1,000 mg/kg.

The term "diagnostically labeled" means that the antibody has attached to it a diagnostically detectable label.

There are many different imaging labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include radioactive isotopes and paramagnetic isotopes.

For diagnostic *in vivo* imaging, the type of detection instrument available is a major factor in selecting a given radionuclide. The radionucleotide chosen must have a type of decay which is detectable for a given type of instrument. In general, any conventional method for visualizing diagnostic imaging can be utilized in accordance with this invention.

Another important factor in selecting a radionuclide for *in vivo* diagnosis is that the half-life of a radionucleotide be long enough so that it is still detectable at the time of maximum uptake by the target, but short enough so that deleterious radiation upon the host is minimized. Ideally, a radionuclide used for *in vivo* imaging

will lack a particulate emission, but produce a large number
of photons in a 140-200 ke V range, which may be readily
detected by conventional gamma cameras.

For *in vivo* diagnosis, radionucleotides may be bound to
antibody or antigen either directly or indirectly by using
an intermediary functional group. Intermediary functional
groups which are often used to bind radioisotopes which
exist as metallic ions to antibody or antigen are
diethylenetriaminepentaacetic acid (DTPA) and
ethlenediaminetetracetic acid (EDTA). Typical examples of
metallic ions which can be bound to immunoglobulins are
^{99m}Tc, ^{123}I, ^{111}In, ^{131}I, ^{97}Ru, ^{67}Cu, ^{67}Ga, ^{72}As, ^{89}Zr, and
^{201}Tl.

The antibodies used in the method of the invention can
also be labeled with paramagnetic isotopes for purposes of
in vivo diagnosis. Elements which are particularly useful
(as in magnetic resonance imaging (MRI) techniques) in this
manner include ^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Cr, and ^{56}Fe.

Preparations of the imaging antibodies for
administration include sterile aqueous or non-aqueous
solutions, suspensions, and emulsions. Examples of non-
aqueous solvents are propyleneglycol, polyethyleneglycol,
vegetable oil such as olive oil and injectable organic
esters such as ethyloleate. Aqueous carriers include water,
alcoholic/aqueous solutions, emulsions or suspensions,
including saline and buffered media, parenteral vehicles
including sodium chloride solution, Ringer's dextrose,
dextrose and sodium chloride, lactated Ringer's or fixed
oils. Intravenous vehicles include fluid and nutrient
replenishers, electrolyte replenishers, such as those based
on Ringer's dextrose, and the like. Preservatives and other
additives may also be present, such as, for example,
antimicrobials, anti-oxidants, chelating agents, and inert
gases and the like. See, generally, *Remington's
Pharmaceutical Science*, 16th ed. Mac Eds. 1980.

Of course, the expressed Bcl-Y protein is an intracellular protein. Accordingly, those of skill will recognize that *in vivo* diagnostic and therapeutic methods employing the antibodies of the invention may require some mechanism by which such antibodies can detect Bcl-Y in the cell. One such method is to introduce the antibodies or fragments thereof into the cell itself across the cell membrane. This may be accomplished, for example, by attaching the antibody to a ligand for which the target cell contains receptor sites. The antibody can thus be transported into the cell membrane or across the cell membrane along with the ligand. Suitable ligands include growth factors and cytokines that are internalized upon receptor binding. Suitable growth factors include epidermal growth factor (EGF), tumor growth factor alpha (TGF-α), fibroblast growth factor (FGF), insulin, and insulin-like growth factors 1 and 2 (TGF-1 and -2). Suitable cytokines include G-CSF, GM-CSF, erythropoietin, IL-1 and IL-2. It is noted that there are also receptors that carry nutrients and vitamins into cells. These nutrients are suitable for use as ligands in the present invention and include foliate, dihdrofoliate, tetrahydrofoliate and vitamin B12.

The choice of a carrier ligand will depend on several factors, as those of skill will appreciate. These include, for example, the kinetics of the ligand and its receptor, and of overall transport, which may include passive or active, with actively transported ligands preferred. The means of attaching the antibody to the ligand also will vary within limits, and may be, for example, covalent or ionic, bearing in mind that such attachment should not unacceptably alter ligand-receptor affinity.

Examples of receptors suitable for such applications include the receptor for low density lipoprotein (LDL), which has been shown to contain all the information necessary for receptor endocytosis, Davis *et al.*, *J. Cell Biol.* 107(6/3): Abstr. No. 3112 (1988), as well as known

brain-specific receptors such as those for dopamine. In this regard, it will be appreciated that the ligand may itself be an antibody or fragment specific for the receptor, to which may be conjugated the antibody of the invention.

5   Moreover, those of skill may find it particularly desirable to employ antibody fragments of the invention (such as, for example, Fab or F(ab')$_2$ fragments), which are less likely to interfere with the ligand-receptor interaction, and may be more easily transported across the

10 cell membrane. Single-chain antibodies may prove preferable for these and other reasons, as will be appreciated by those of skill.

When an antibody is to be transported into the cell's membrane or into the cell as described above, it will be

15 preferred to diagnostically or therapeutically label the antibody in such a way that the label will be relatively more effective when the antibody is bound to its antigenic site on the Bcl-Y protein. This may accomplished, for example, by employing a label which becomes active or

20 detectable as a result of formation of the antigen-antibody complex. Alternatively, the antibody itself may be labeled in such a way that antigen-antibody complex formation induces a conformational change in the antibody to expose or more fully expose the previously unexposed or less fully

25 exposed label. All of the above criteria, and others, will be apparent to those of skill in carrying out these aspects of the invention.

It is also possible to utilize liposomes having the antibodies of the present invention in their membranes to

30 specifically deliver the antibodies to the target area. These liposomes can be produced so that they contain, in addition to the antibody, such therapeutic agents as drugs, radioisotopes, lectins and toxins, which would act at the target site.

35

V.   Pharmaceutical Compositions

Pharmaceutical compositions containing a therapeutically effective amount of the present Bcl-Y protein, functional equivalents, fragments and/or hybrids and/or mutants thereof, as well as vectors containing cDNA encoding one or more of the foregoing, are useful for treating patients suffering from degenerative disorders characterized by inappropriate cell death or inappropriate cell proliferation.

Hybrids of Bcl-Y include hybrids of Bcl-Y and for example Bcl-2, ced-9, Bcl-X, Bcl-X_L, Bcl-X_S, Bax, Mcl, c-myc, LMW5-HL, BHRF-1 and A1. Such hybrids exhibit enhanced, decreased or intermediate apoptosis induction or suppression activity as compared to the activity of Bcl-Y alone. These hybrids can be readily selected, produced and employed by one or ordinary skill in the art. Pharmaceutical compositions according to the invention thus will contain a therapeutically effective amount of the present Bcl-Y protein, functional equivalents, fragments and/or hybrids and/or mutants thereof, and may optionally contain one or more pharmaceutically acceptable carriers and/or excipients, known to those of ordinary skill in the art. Administration, dosage and frequency, and length of the course of treatment can be readily optimized for a particular patient by one of ordinary skill in the art. For example, the present pharmaceutical composition can be formulated as sterile aqueous or non-aqueous suspensions or emulsions, as described above in Section IV, for example for solutions for intravenous administration.

VI.   Therapeutic Applications

Programmed cell death is a process in which cells undergo nuclear condensation and fragmentation during normal development of healthy tissues and organs. The process is

essential in maintaining the balance between growth of new cells and elimination of old cells. When apoptosis does not work properly, either by causing cells to die prematurely or by preventing them from dying when scheduled, various disorders develop.

The present apoptosis associated Bcl-Y protein, functional equivalents, fragments and/or hybrids and/or mutants thereof as well as vectors containing cDNA encoding the foregoing are useful for treating degenerative

10 disorders, which disorders are characterized by inappropriate cell death or inappropriate cell proliferation. Particular disorders may involve different cell types whereby it may be desirable to induce apoptosis in one cell type while suppressing apoptosis in the other.

15 For example, it may be desirable to suppress apoptosis in lung tissue cells in a patient suffering from acute lung injury by administering the Bcl-Y mutant protein of the invention (or by effecting expression of such Bcl-Y mutant protein in those cells) while inducing apoptosis in

20 fibroblast cells which may be present in the lung due to the inflammatory response by administering the Bcl-Y protein of the invention (or by effecting Bcl-Y protein expression in those cells).

The therapeutic agents of the present invention can be

25 administered as discussed above with the requirement that the agent must cross the cell membrane. The therapeutic agent can be administered alone, in combination with or during the course of treatment with other acceptable therapies known in the art for treating a particular

30 disorder. For example, the present therapeutic agents can be administered to induce apoptosis in a cancer patient who is also undergoing classic cancer therapy including, for example, radiation therapy, chemotherapy, and treatment with anti-cancer drugs including, for example, topoisomerase

35 inhibitors, alkylating agents, antimetabolites, and hormone antagonists. Further, the present therapeutic agents can

also be administered concurrently with gene therapy. For
example, the present therapeutic agents can be administered
to a patient suffering from a degenerative disorder of the
central nervous system while the patient is concurrently
5 undergoing gene therapy to replenish neutrophic hormones.

Premature widespread apoptosis (inappropriate cell
death) causes much of the damage associated with
degenerative disorders including, for example, AIDs,
chemotherapy and radiation, and tissue atrophy. In AIDs
10 patients, lymphocytes are activated even in the asymptomatic
phase of the HIV infection, and those cells die prematurely
by apoptosis. Such disorders may admit of treatment by
administration of a Bcl-Y mutant protein.

Those of skill will appreciate that administration of
15 the various proteins of the invention to particular target
cells or tissues, as described herein, is intended to
comprehend the administration of the proteins themselves as
well as the expression by the target cells or tissues of the
nucleotide sequences encoding those proteins by various
20 known means and in accordance with the teachings of the
present specification.

Degenerative disorders characterized in inappropriate
cell proliferation include cancer, autoimmune disorders,
tissue hypertrophy, and inflammatory disorders including
25 inflammation arising from acute tissue injury including, for
example, acute lung injury. These disorders can be treated
by administering the present Bcl-Y protein or functional
equivalent.

Cancers arise when changes in DNA cause the anomalous
30 accumulation of cells. The comparative rates of cell
division and cell deaths determine how fast a cancer grows.
Some cancer cells divide more slowly than normal cells, but
the cancer may still expand because of prolonged cell life
span. Apoptosis is an efficient method for preventing
35 malignant transformation because it removes cells with
genetic lesions. Defective apoptosis can promote cancer

development, both by allowing accumulation of dividing cells and by obstructing removal of genetic variants with enhanced malignant potential. The present therapeutic agents, including the present Bcl-Y protein, functional equivalents,

5   fragments, and hybrids thereof along with vectors containing cDNA encoding the one or more of the foregoing, can be administered to cancer patients to induce apoptosis.

Many types of cancer can be treated by the administration of the present therapeutic agents, including

10   for example, carcinomas, sarcomas, and leukemia/lymphomas, including for example, carcinomas such as adenocarcinomas, squamous carcinomas, carcinoma of the organs including breast, colon, head, neck, etc.; sarcomas including chondrosarcoma, melanosarcoma, etc.; and leukemia and

15   lymphomas including acute lymphomatic leukemia, acute myelogenous leukemia, non-Hodgkin's lymphoma, Burkitt's lymphoma, B-cell lymphomas, T-cell lymphomas, etc. Other conditions amenable to treatment using the present therapeutic agent include fungal infections.

20   The present therapeutic agents can be used to treat autoimmune diseases. Random gene recombination and somatic hypermutation can potentially generate autoreactive T and B lymphocytes throughout life. Under normal conditions immature lymphocytes that bind autoantigens die by

25   apoptosis. However, a defect in the deletion of these lymphocytes predisposes one to autoimmunity.

The present therapeutic agents can be administered to patients suffering from autoimmune disorders to induce apoptosis in autoreactive T lymphocytes, for example, in

30   patients suffering systemic lupus erythematosus. Other autoimmune diseases amenable to treatment by suppressing or inducing apoptosis through the administration of the present therapeutic agents include, for example, rheumatoid arthritis, myasthenia gravis, Grave's disease, Hashimoto's

35   thyroiditis, insulin-resistent diabetes, allergic rhinitis, asthma, functional autonomic abnormalities, juvenile

insulin-dependent diabetes, Addison's disease, idiopathic
hypoparathyroidism, spontaneous infertility, premature
ovarian failure, pemphigus, Bullous pemphigoid, primary
biliary cirrhosis, autoimmune hemolytic anemia, idiopathic
5 thrombocytopenic purpura, idiopathic neutropenia,
Goodpasture's syndrome, rheumatoid arthritis and Sjogren's
syndrome.

     The present therapeutic agents can be used to treat
inflammation resulting from acute lung injury, by inducing
10 apoptosis. The disease process begins with an explosive
inflammatory response in the alveolar wall. In the
aftermath of the resulting tissue destruction, extensive
fibroproliferation of the alveolar air space ensues, ·
consisting of fibroblasts, capillaries and their connective
15 tissue products. Fukuda, Y., et al., Am. J. Pathol.
126:171-182 (1987). An important mechanism for the
systematic elimination of the foregoing is apoptosis, i.e.,
programed cell death.

     The present therapeutic agents can also be used to
20 treat degenerative disorders due to premature or excessive
cell loss during aging which can lead to organ disfunction
and disease. Such degenerative disorders include
degenerative diseases of the central nervous system due to
aging or other factors which result in the death of neurons.
25 The present therapeutic agents containing Bcl-Y mutant
protein or hybrids thereof can be administered to a patient
suffering from such a degenerative disorder to suppress
apoptosis. Further, the present therapeutic agents can be
administered concurrently with gene therapy to provide genes
30 encoding neutrophic hormones including, for example, nerve
growth factor. Other conditions amenable to treatment
utilizing the present therapeutic agents include, for
example, Alzheimer's disease.

     One of ordinary skill in the art can readily identify
35 other degenerative disorders characterized by inappropriate
cell death or inappropriate cell proliferation or both which

are amenable to treatment using the present therapeutic
agents. The present therapeutic agents can include the
Bcl-Y protein itself, as well as fragments, functional
equivalents and/or hybrids and/or mutants thereof, which are
5 administered to a target cell. Alternatively, therapeutic
agents according to the invention can be administered by
infecting the target cell with a vector containing cDNA
encoding one or more of the foregoing. The present
therapeutic agents can be administered to the desired target
10 cell as discussed below, for example, by choosing a receptor
on the target cell surface which is specific for that cell
type. The present therapeutic agents can be administered
alone or in combination with other acceptable drug
therapies. Further, the present therapeutic agents can be
15 administered concurrently with other acceptable therapies
specific for the particular degenerative disorder being
treated. For example, the present therapeutic agents can be
administered concurrently with chemotherapeutic agents, gene
therapy, or the like. Whether it is the Bcl-Y protein
20 itself or the a vector encoding the protein, the therapeutic
agent must cross the cell membrane.

        One method for introducing the Bcl-Y protein or
fragments thereof into the cell's membrane or into the cell
itself is by attaching the protein to a ligand for which the
25 target cell contains receptor sites. The protein can thus
be transported into the cell membrane or across the cell
membrane along with the ligand.

        The choice of a carrier ligand will depend on several
factors, as discussed herein and known to those of skill.
30 Suitable tissue-specific receptors include: Brain: nerve
growth factor receptor (NGF-R); breast: prolactin receptor;
stomach: gastrin receptor; skin: melanocyte stimulating
hormone receptor (MSH-R), liver: asialoglycoprotein
receptor; thyroid: thyroid stimulating hormone receptor
35 (TSH-R); ovaries: luteinizing hormone receptor (LH-R),
testis: human chorionic gonadotrophin receptor (hCG-R),

T-cells: T-cell receptors; B cells: CD19; lung hyaluronate
receptor CD44 isoform 4V (J. Cell. Biol. 124, 7182, 1994).
In this regard, it will be appreciated that the ligand may
be an antibody or fragment specific for the receptor, to
which may be conjugated the Bcl-Y protein of the invention.

It may be desirable to employ active Bcl-Y fragments
according to the invention which are less likely to
interfere with the ligand-receptor interaction, and which
may be more easily transported across the cell membrane.

10 When a protein is to be transported across the cell's
membrane or into the cell as described above and the ligand
is an antibody, it will be preferred to diagnostically or
therapeutically label the protein in such a way that the
label will be relatively more effective when the protein is

15 bound, such as, for example, by means analogous to those
described herein in the context of antibody transport.

It is also possible to utilize liposomes having the
proteins of the present invention in their membrane to
specifically deliver the present Bcl-Y proteins to the

20 target area. These liposomes can be produced so that they
contain, in addition to the Bcl-Y protein, such other
therapeutic agents including drugs, radioisotopes, lectins
and toxins, which would be released at the target site.

A preferred manner for administering the Bcl-Y encoding

25 nucleotide sequences (and their functional equivalents and/
or hybrids and/or mutants) for diagnostic or therapeutic
purposes is by the use of viral vectors. Suitable viral
vectors for gene transfer include retroviruses (reviewed in
Miller, et al., Methods Enzymol. vol. 217, p.581-599 (1993))

30 including human immunodeficiency virus (HIV), adenovirus
derivatives (for examples see Erzurum, et al. Nucleic Acids
Res. Vol. 21, p.1607-12 (1993); Zabner, et al., Nat. Genet.
Vol. 6, p.75-83 (1994); Davidson, et al., Nat. Genet. vol.
3, p.219-223 (1993)) adeno-associated virus (AAV), (i.e. see

35 Flotte, et al., Proc. Natl. Acad. Sci. vol. 90, p.10613-7
(1993)) and Herpes virus vectors (i.e. see Anderson, et al.,

Cell Mol. Neurobiol. vol. 13, p.503-15 (1993)). Other
suitable viruses can be readily selected and employed by
those of ordinary skill in the art. Other methods for DNA
delivery include liposome mediated gene transfer (Alton, et
al., Nat. Genet. vol. 5, p. 135-42 (1993); Nabel, et al.,
Proc. Natl. Acad. Sci USA vol. 90, p. 11307-11 (1993)).

The use of viral vectors for introduction of genes into
mammalian cells is also reviewed, for example, in Varmus,
Science 240(4858):1427 (1988); Eglitis et al., BioTechniques
6,7:608 (1988); Jaenisch, Science 240(4858):1468 (1988); and
Bernstein et al., Genet. Eng. (N.Y.) 7:235 (1985).

For the purposes of the present invention, it may be
preferred to employ an attenuated viral or retroviral
strain. Thus, for example, it is possible to use as vectors
for the DNA sequences of the invention retroviruses having
attenuated cytopathicity, such as HIV-2$_{ST}$ (Kong et al.,
Science 240(4858):1525 (1988)) or HIV-2$_{UC1}$ (Evans et al.,
Science 240(4858):1523 (1988)), which enter neural cells by
a CD4-dependent mechanism (Funke et al., J. Exp. Med.
165:1230 (1987)). The neurobiology of HIV infections is
described, for example, in Johnson et al., FASEB J.
2(14):2970 (1988). Those of skill will be able to target
different cell populations having known susceptibilities to
viruses by the exercise of routine skill. For example, CD4
is known to have a variant transcript in the human brain,
with its highest content in forebrain (Maddon et al., Cell
47:333 (1986). Possible methods to target retroviral gene
expression to specific cell types are reviewed by
Boris-Lawrie and H. Temin Curr. Opin. Genet. Dev. vol. 3,
p.102-9 (1993).

Ideally, then, the choice of a gene delivery system
will be made by those of skill, keeping in mind the
objectives of efficient and stable gene transfer, with an
appropriate level of gene expression, in a
tissue-appropriate manner, and without any adverse effects.
See, for example, Wolff et al., Rheum. Dis. Clin. North Am.

14(2):459 (1988). With respect to delivery to a central
nervous system target, many viral vectors, including HIV,
offer the advantage of being able to cross the blood-brain
barrier (Johnson *et al.*, *FASEB J. 2(14)*:2970 (1988)).

VII. Diagnostic Applications

Antibodies raised against the present Bcl-Y protein,
fragments, functional equivalents, or hybrids or mutants
thereof can be used to detect the Bcl-Y protein in a human
tissue sample, as well as to diagnose degenerative disorders
associated with the expression of the Bcl-Y protein.
Further, such antibodies can also be used to monitor the
progress of degenerative disorders associated with the
expression of the Bcl-Y protein.

Any source of human cells is suitable for use in the
diagnostic testing in the present invention. The cells can
be isolated from any human tissue including for example,
heart, lung, tumor cells, brain, placenta, liver, skeletal
muscle, kidney and pancreas. Extraction of proteins from
the cell sample may be performed by any of the many means
known in the art. For example, cells may be lysed by a
detergent by mechanical means. If desired, nucleic acids
can be removed from the cell preparation by enzymatic
digestion or by precipitation with agents such as
streptomycin. Such means are well known in the art.

Antibodies can be generated which are immunoreactive
with the Bcl-Y proteins by the methods set forth herein.
Appropriate antibodies can then be screened using the
natural gene products of Bcl-Y.

The extracted proteins from the cell sample may be
contacted with the antibody under suitable conditions for
antibody-antigen complex formation. Generally, such
conditions are physiological conditions. The protein
extract may be bound to a solid support such a nitrocellular
filter or a microtiter plate.

The antibody will generally bear a label which is a radio label, a florescent label, or an enzyme conjugate which under appropriate conditions produces, for example, a colored reaction product. Antibodies and antibody labeling

5 are described herein and known to those of skill. Alternatively, if the antibody is not labeled, it can be detected by means of a second antibody from another species which is reacted with the first antibody. Suitable assay techniques, labels and means of detection are discussed

10 herein.

A parallel sample to the test sample is employed to provide the control. The control sample consists of an equivalent amount of proteins extracted from cells, preferably in the same manner as those of the test sample.

15 The amount of protein can readily be determined by employing techniques well known in the art, including, for example, the Lowry or Bradford techniques. The cells used for preparing the control sample may be selected from cells of the same cell type as the test cells, isolated from a normal

20 human not suffering from the degenerative disorder from which the human from which the test sample was taken suffers, cells of the same cell type as the test sample isolated from an established normal cell line, and cells from the human who is being tested, which cell type is

25 different from the cell type of the test cells.

Test samples can also be screened for elevated levels of mRNA transcribed from the Bcl-Y gene, according to methods well known in the art. For example, RNA extracted from B-cells may be used, or alternatively mRNA may be

30 isolated from total cellular RNA. The mRNA may be purified, for example, by affinity chromatography on oligo (dT cellulose) which binds to the poly (A) tract at the 3' end of most mRNA. As is well known to those skilled in the art, it is essential that ribonuclease activity be minimized

35 during preparation and assaying.

A DNA probe may be selected from any of the protein coding sequences of the Bcl-Y gene. Preferably, the probe will be selected from sequences of the 5' or 1st exon of the gene, so that all three species of RNA can be detected.

5   Preferably, the probe contains at least 15 nucleotides of the Bcl-Y sequence. In order to perform the hybridization, it is desirable that the probe be single stranded. Thus, if the probe is double stranded, it should be denatured to a single stranded form. Means for denaturing are well known

10 in the art, including alkali or heat treatment. The probe can then be contacted with the RNA derived from the cell sample under conditions where homologous RNA-DNA hybrids form and are stable. Such conditions are well known in the art. Means for detecting hybrids are many and well known,

15 but often involve the use of radiolabeled probes and nucleases which degrade single stranded DNA. Other methods known in the art may be used.

Control samples can be derived from any of these cell sources described above for use in the antibody diagnostic

20 tests. Samples and controls should preferably be prepared in parallel under similar conditions.

The diagnostic methods and compositions of the present invention are useful for determining whether a disease/ degenerative disorder is linked to abnormal Bcl-Y

25 expression, as well as for determining the effect of over expression or loss of expression of Bcl-Y in animal models such as transgenic mice and/or homozygous null mice. Methods for determining whether a disease/degenerative disorder is linked to abnormal Bcl-Y expression include

30 analyzing Bcl-Y expression in diseased tissue as compared to normal tissue by for example, Northern and/or Western blots, as well as by other assay methods readily chosen and employed by those of ordinary skill in the art. Once it has been determined that a disease/degenerative disorder is

35 linked to abnormal Bcl-Y expression, the disease/disorder can be diagnosed in an individual.

The following Examples are offered by way of illustration, not by way of limitation.

Examples

Example 1

A.   Materials and Methods

1.   **Degenerate PCR.** Poly A+ RNA was prepared from
10 total cellular RNA by oligo dT chromatography using the PolyATract system (Promega) following the manufacturers protocol. Approximately 0.5 μg of poly A+ RNA was converted to cDNA using the Gibco/BRL (MD) cDNA synthesis pre-amplification system. The reverse transcription reactions
15 were primed with random hexamer oligonucleotides. Two degenerate oligonucleotide primers were synthesized with the following sequences:

BD2:   5'-CGGAAGATCTAA(C,T)TGGGG(N)(A,C)G(N)(A,G)T(N)GT-3'
[SEQ ID NO: 1]

20 BU3:   5'-GCTTCTGCAG(A,G)AA(N)(C,G)C(N)TCCCA(N)CC(N)CC-3'
[SEQ ID NO: 2]

PCR reactions contained the following components: approximately 100 ng of cDNA template, 12 μM of each primer (BD2 and BU3), 1.5 mM MgCl2, 0.2 mM dNTP, 50 mM KCl, 10 mM
25 Tris-HCL [pH 8.3], and 2.5 units Taq polymerase (Perkin Elmer) in a total volume of 50 μl. The reaction was denatured at 94° for 5 minutes followed by two amplification cycles at low annealing temperature consisting of: 94° 1 min., 40° 1 min., and 72° 1 min. Thirty
30 additional cycles were subsequently performed at normal annealing temperature: 94° 1 min., 55° 1 min., 72° 0.5 min. The PCR reactions were precipitated with ethanol, resuspended and digested with Bgl II and Pst I which cut at the ends of primers BD2 and BU3, respectively. The digested
35 PCR products were then electrophoresed on a 9% polyacrylamide Tris-borate-EDTA gel. DNA fragments of the

expected size (around 200 bp) were excised and purified by
electrophoretic transfer to NA45 membranes (Schleicher and
Schluell). The purified DNA was cloned into pBluescript KS
(Stratagene), and individual clones were analyzed by DNA
sequencing using the Sequenase system (US Biochemicals).

    2.   Isolation of cDNA clones. The 200 bp Bcl-Y
partial cDNA fragment was 32-P labeled by the random hexamer
priming method to a specific activity >10^8 cpm/μg. This
probe was then used to screen a Lamda ZAP II (Stratagene)
Jurkat cDNA library. Positive phage were plaque purified by
secondary and tertiary screening, and cDNA inserts in
positive lamda clones were rescued as plasmids by in vivo
phagemid excision following the protocol supplied by
Stratagene. Clones were further analyzed by restriction
enzyme site mapping and DNA sequencing. A portion of the
cDNA sequence extending 5' of the Bcl-Y open reading frame
was isolated by the amplification of cDNA ends PCR method
(Frohman, M.A., et al., *Proc. Natl. Acad. Sci. USA*
85:8998-9002 (1988)), using the 5'-Amplifinder RACE system
(Clontech).

    3.   In vitro translation. 35-S labeled proteins were
synthesized in vitro using the TnT T7/T3 coupled
reticulocyte lysate system (Promega), following the
manufacturer's procedures.

    4.   Western blot analysis. COS7 and NIH3T3 cells were
cultured in DMEM supplemented with 10% fetal calf serum, and
L-glutamine. Cells were transfected with plasmid DNAs
(typically 2 to 8 μg) using Lipofectamine (Gibco/BRL), and
cell lysates were prepared 48 hours after transfection.
Portions of the cell extracts (approximately 100 μg) were
electrophoresed on SDS polyacrylamide gels, and transferred
to nylon membranes by standard methods (Harlow, E., et al.,
Antibodies: A Laboratory Manual (1988)). Blots were
incubated with the anti-HA epitope monoclonal antibody 12CA5
(Kolodziej, P.A., et al., *Meth. Enzymol. 194*:508-519

(1991)), which was subsequently detected with a secondary antibody using the ECL system (Amersham).

5.   **Plasmid constructions.** All DNA manipulations were performed following standard protocols (Sambrook, J., et

5 al., *Molecular Cloning: A Laboratory Manual* (1989)). The Bcl-Y coding sequence was amplified by PCR using synthetic oligonucleotide primers which introduce BamHI and EcoRI sites at the 5' and 3' end, respectively, of the Bcl-Y reading frame. The DNA sequence of the primers is as

10 follows: 5'-GCAGGATCCGCTTCGGGGCAAGGCCCAG-3' [SEQ ID NO: 3] and 5'-GCGGAATTCGAGTCATGATTTGAAGAATC-3' [SEQ ID NO: 4]. The Bcl-Y PCR product was digested with BamHI and EcoRI and cloned into the BamHI/EcoRI sites of a pcDNA1/amp derivative (Krek, W., et al., *Science 262*:1557-1560 (1993)) containing

15 a short sequence coding for the hemagglutinin epitope. The correct DNA sequence of the Bcl-Y PCR product was confirmed by DNA sequencing. The resulting construct contains amino acids 2 to 211 of Bcl-Y fused, in frame, to the HA tag encoding peptide sequence: MAYPYDVPDYASLGS [SEQ ID NO: 5].

20 The HA-Bcl-Y protein is expressed from the CMV enhancer/ promoter sequences provided by the pcDNA-1/amp backbone vector (Invitrogen).

Bax and Bcl-X coding sequences were isolated by PCR amplification of Namalwa cell cDNA using primers based on

25 the published cDNA sequences (Boise, L.H., et al., *Cell 74*:597-608 (1993); Oltvai, Z.N., et al., *Cell 74*:609-619 (1993)). The PCR products were initially cloned into pBluescript and sequenced to confirm that the clones contained the wild type Bax and Bcl-X DNA sequences. HA

30 epitope tagged forms of Bax and Bcl-X were generated by subcloning into the pcDNA1/amp derivative described above. A human Bcl-2 cDNA cloned into pBluescript was obtained from Dr. Gerard Evan (ICRF, London).

6.   **Northern blot analysis.** PolyA+ RNA from multiple

35 tumor cell lines was prepared using the FastTrack mRNA isolation system (Invitrogen) following the manufacturer's

protocol. Northern blot analysis was performed by standard
methods (Sambrook, J., et al., *Molecular Cloning: A
Laboratory Manual* (1989)). The tumor cell line Northern
blots were hybridized to a 32-P labeled probe derived from
5   the 200 bp Bcl-Y PCR product. A human multiple tissue
Northern blot was purchased from Clontech laboratories and
hybridized sequentially to 32-P labeled probes encompassing
the entire coding regions of Bcl-Y, Bcl-2 and Bax, and ß-
actin following the supplier's protocol.

10   7.   **IL-3 deprivation.** IL-3 deprivation studies were
performed essentially as described (Hockenbery, D., et al.,
Nature 348:334-336 (1990)). Briefly, cells were cultured
for 24 hr in the presence of IL-3, washed extensively, and
seeded in 24 well plates at 5 x10^5 cells/ml in media lacking
15 IL-3. Viability was determined by counting live and dead
cells after trypan blue staining.

Recombinant retroviruses carrying HA-Bcl-y and HA-Bax
were generated by cloning the relevant cDNAs into the pBabe
Puro vector (J. P. Morgenstern, H. Land, *Nucl. Acids Res.*
20 **18**, 3587-3596 (1990)) and transfecting the WE packaging cell
line. Supernatants from the resulting producer cell lines
were used to infect FL5.12 cells, and infected cells were
selected in media containing 1 µg/ml puromycin.

8.   **In vitro protein interactions.** Glutathione S-
25 transferase fusion proteins of bcl-2 (GST-bcl-2) and its
homolog bcl-xl (GST- bcl-xl) were expressed in E. coli and
purified by affinity chromatography using glutathione-
agarose (Smith, D.B. and Johnson, K.S., Gene 67, 31 (1988).
35S-Methionine-labeled HA-bcl-y was expressed in vitro using
30 a coupled transcription/translation system in rabbit
reticulocyte lysates (Promega). Labeled HA-bcl-y (1-10 nM)
was precleared with 10% glutathione-agarose in 10 mM HEPES
buffer, pH7.2 containing 0.25% NP-40, 142.5 mM KCl, 5 mM
MgCl2 and 1 mM EGTA (Buffer A). GST fusion proteins were
35 added (final concentration 1-3 mM) and the mixtures
incubated at 37°C for 5 min., followed by 60 min. at 4°C.

Protein complexes were captured with 10% glutathione-agarose
and washed twice with Buffer A and twice with Buffer A
without NP-40. Proteins were eluted from the beads by
incubation in SDS-PAGE sample buffer at 100°C for 5 min and
5 loaded onto 4-20% SDS-polyacrylamide gels. Following
electrophoresis, gels were fixed and incubated in a
fluorography enhancing solution. (Amplify; Amersham). The
gels were dried and subjected to autoradiography at -70°C.

10 **B.   Results**

   1.   **Cloning of a partial Bcl-Y cDNA by degenerate PCR.**

   It was anticipated that additional, as yet
15 undiscovered, Bcl-2 family members would likely contain
amino acid segments closely resembling domains I and II.
The identification and cloning of novel Bcl-2 relatives by
PCR using primers based on these homologous domains, was
attempted. A series of oligonucleotide primers were
20 synthesized with sequences corresponding to variations of
the most conserved amino acids present in domains I and II.
The primers contained appropriate DNA sequence degeneracies
so that all possible triplet codons for a given amino acid
residue were encoded. One particular set of degenerate
25 primers, BD2 and BU3 (see Fig. 1A), were designed to anneal
to any cDNA(s) coding for the domain I and II segments
NWGR(I,V,M)V, and GGW(E,D)(A,G)F, respectively. Restriction
enzyme sites (BglII or PstI) were incorporated into the 5'
ends of the primers to facilitate cloning of PCR products.
30   The human B-cell lymphoma cell line, Namalwa, was
initially chosen as a source of cDNA for PCR experiments.
It was expected that these cells would express Bcl-2,
thereby providing a positive control to ensure that the
degenerate primers were capable of amplifying Bcl-2 related
35 cDNA(s). RNA (poly A+) was prepared from Namalwa cells, and
converted to cDNA by reverse transcription. The cDNA was

then used as template DNA in a PCR reaction containing
oligonucleotides BD2 and BU3. Two cycles with low annealing
temperature (40 deg.) were performed to permit a small
degree of mismatching between the primers and template to
5   increase the effective degeneracy of the primers. This was
followed by 30 amplification cycles at a 55 degree annealing
temperature. The PCR reactions were digested with BglII and
PstI, and electrophoresed on a 9 % acrylamide gel. Since
the spacing between domains I and II is well conserved
10   (Fig.1A), the PCR products in the expected size range
(around 200 bp) were purified and cloned into a plasmid
vector for DNA sequence analysis.

     The DNA sequence of eighteen independent clones was
determined. Three of the clones were cDNAs clearly
15   unrelated to Bcl-2 and were amplified due to fortuitous
homology to the degenerate primers. The majority of the PCR
clones, however, appeared to be bona fide Bcl-2 related
cDNAs. One isolate each of Bcl-2 and Bcl-X$_L$ was obtained
and seven clones were identical to Bax. Six of the clones
20   appeared to be a novel Bcl-2 related gene, which was
designated "Bcl-Y" since it was isolated by virtue of its
homology to Bcl-2. The conceptual translation of the Bcl-Y
cDNA segment cloned by PCR indicates that it is an authentic
Bcl-2 relative (Fig. 1B). An open reading frame was
25   maintained between the two PCR primers in the appropriate
register, and several key amino acids flanking domains I and
II that are also highly conserved among known Bcl-2 family
members (Williams, G.T., *Cell* 74:777-779 (1993)), were
present at the identical positions in the Bcl-Y sequence
30   (Fig 1B).

     The PCR cloning procedure was repeated with cDNA
prepared from the human leukemia cell line U937. In this
experiment, two of eighteen PCR clones isolated were Bcl-Y.
Subsequent PCR experiments with cDNA from breast, colon and
35   ovarian carcinoma cell lines also yielded substantial
numbers of Bcl-Y clones. Thus, Bcl-Y partial cDNA clones

could be readily isolated from diverse human tumor cell lines by this method.

2.   Expression of Bcl-Y in human tumor cell lines.

Northern blot analysis was performed in order to determine the size of the Bcl-Y mRNA and to examine its range of expression in human tumor cells. The partial Bcl-Y cDNA cloned by PCR was labeled with 32P and used as a probe in Northern blots containing poly A+ RNA from a number of different tumor cell lines. The probe hybridized to a 2.2 kb RNA species in all cell lines examined (Fig.2), including Namalwa, U937, Jurkat (T-cell, data not shown), several breast carcinoma cell lines (MCF7, BT549, MBAMD468), the colon carcinoma cell lines HT29 and SW116, and the ovarian carcinoma cell line OVCAR3. These results demonstrate that the 2.2 kb Bcl-Y mRNA is expressed in a wide variety of human tumor cell lines.

3.   Isolation of a full length Bcl-Y cDNA.

The cDNA clone isolated by degenerate PCR represented a partial segment (~ 200 bp) of the predicted 2.2 kb full length Bcl-Y cDNA based on the size of the mRNA on Northern blots.   In order to isolate cDNA clone(s) encoding the entire Bcl-Y protein, a Jurkat cell cDNA lambda phage library was screened using the PCR-derived partial cDNA clone as a probe. Three overlapping cDNA clones were obtained that together span about 2.0 kb of the predicted 2.2 kb Bcl-Y full-length cDNA (lambda clones 4, 10, 11, Fig. 3). DNA sequence analysis indicated that lambda clone 11 encompassed the entire Bcl-Y open reading frame (Fig 3). Additional 5' non-coding sequence (about 200 bp) was cloned by the rapid amplification of cDNA ends (RACE) PCR method. The size of the composite cDNA assembled from these various

overlapping clones is approximately 2.2 kb indicating most,
if not all, of the Bcl-Y cDNA has been isolated.

The composite cDNA sequence derived from the various
overlapping clones, and the conceptual translation of the
Bcl-Y open reading frame, is shown in Figure 4.   A
methionine codon (ATG), located near the 5' end is predicted
to function as the initiating methione since it is preceded
by translational stop codons (in all three reading frames),
and resembles the consensus initiation sequences described
by Kozak (Kozak, M. *Nucl. Acids Res.* *15*:8125-8132 (1986)).

4.   Bcl-Y is homologous to Bcl-2 family members.

The complete Bcl-Y amino acid sequence was compared to
the peptide sequence database (Feb. 10, 1994 release) using
the BLAST network service provided by the NCBI.   Bcl-Y
showed the highest degree of homology to the human Bcl-2
protein.   Bcl-Y exhibited 43% identity to the human Bcl-2
protein over a 54 amino acid section (residues 82 to 136)
encompassing domain I (Fig.5).   An 20 amino acid stretch
containing domain II (residues 164 to 184), and a 20 residue
segment near the N-terminus (residues 54 to 73) showed 40%
and 45% identity, respectively, to Bcl-2 alpha (Fig.5).

Alignment of the deduced Bcl-Y amino acid sequence with
the known family of Bcl-2 related proteins is shown in
Figure 6.   The highest degree of homology is centered around
domains I and II, as is the case with the known Bcl-2
relatives (Fig.6).   Certain amino acids outside of these
domains are highly conserved between the previously
identified Bcl-2 homologues (Williams, G.T., *Cell*
65:1097-1098 (1991)).   These same residues are also present
in the Bcl-Y sequence at analogous positions (indicated by
the shaded residues, Fig 6).   An additional feature
generally shared by Bcl-2 family members is the presence of
a C-terminal hydrophobic amino acid domain that provides
membrane localization function (underlined residues, Fig.6).

Bcl-Y also contains a similar stretch of hydrophobic residues at its C-terminus. In addition, the hydrophobic stretch ends in each case with one or more basic residues (bold letters, Fig. 6). The location and extent of primary sequence homology, as well as additional structural similarities (MW and the presence of a C-terminal membrane localization sequence) indicates that Bcl-Y is an authentic novel member of the Bcl-2 family.

5.   **Expression of the Bcl-Y protein** *in vitro* **and in transfected cells.**

The deduced amino acid sequence of Bcl-Y predicts a protein of MW 23.4 kD. *In vitro* translation of lambda clone 11 in rabbit reticulocyte lysates produces a protein with electrophoretic migration on SDS polyacrylamide gels corresponding to a MW of about 26 kd (Fig.7A), in approximate agreement with the size predicted from conceptual translation of the cDNA sequence.

The Bcl-Y protein could also be expressed in transiently transfected cells. In order to detect the protein, the Bcl-Y open reading frame was fused at the amino terminus to a 9 amino acid segment derived from the influenza hemaglutinen antigen (HA). This short peptide provides a well characterized epitope that permits immunological detection of the "tagged" protein by the monoclonal antibody 12CA5 (Kolodziej, P.A., et al., Meth. Enzymol. 194:508-519 (1991)). The HA-tagged Bcl-Y open reading frame was cloned into a CMV based eukaryotic expression vector, and transfected into COS7 and NIH3T3 cells. Cell lysates were prepared 48 hrs after transfection, and analyzed by Western blot with the anti-HA monoclonal antibody. The HA-tagged Bcl-Y protein, of the appropriate size, was detected in both the COS7 and NIH3T3 cell extracts (Fig.7B). These results demonstrate that the

protein encoded by the isolated Bcl-Y cDNA can be expressed both *in vitro* and *in vivo*.

6.   Expression of Bcl-Y in human tissues.

To determine the normal tissue distribution of Bcl-Y gene expression, a northern blot containing RNA from eight different human tissues was hybridized to a probe encompassing the entire Bcl-Y open reading frame. A single 2.2 kb Bcl-Y mRNA was detected in all tissues examined (Fig. 8), suggesting that alternatively spliced Bcl-Y messages, if any, must be present at much lower levels. Heart tissue was found to express the highest levels of Bcl-Y message (when normalized to the levels of ß-actin). The northern blot was sequentially re-hybridized to probes for Bcl-2 and Bax (also shown in Fig.8). These results demonstrate that Bcl-Y has a pattern of expression distinct from these known Bcl-2 family members and its ubiquitous expression indicates that Bcl-Y regulates apoptosis in a broad range of tissues.

7.   Expression of Bcl-Y accelerates apoptosis in transfected cells.

To further test the function of Bcl-y in regulating apoptosis, the effect of Bcl-y expression in FL5.12 cells was investigated. A nine amino acid epitope from the influenza virus hemagglutinin antigen (HA) was engineered at the amino-terminus of Bcl-y to permit detection of the protein by the anti-HA monoclonal antibody 12CA5 (Kolodziej, P.A., et al., Meth. Enzymol. 194:508-519 (1991)). FL5.12 cells were transfected with a plasmid that directs the expression of HA-tagged Bcl-y from the CMV enhancer/ promoter. Transfected cells were selected by virtue of their resistance to the drug G418, and expression of the HA-tagged Bcl-Y protein was detected by Western blot analysis with the anti-HA antibody 12CA5 (Figure 9B). Several FL5.12

clones that expressed the highest levels of HA-Bcl-y were
examined for viability under conditions of IL-3 deprivation,
as previously described (Hockenbery, D., et al., Nature
348:334-336 (1990)). The FL5.12 clones expressing HA-Bcl-y
5 (yb2, yb3, yb4) died at an increased rate relative to an
FL5.12 clone transfected with the expression vector carrying
only the neomycin resistance gene (Figure 9A). Thus, in
marked contrast to the protective effect of Bcl-2 expression
in this cell line (Hockenbery, D., et al., Nature 348:334-
10 336 (1990)), clones expressing Bcl-Y exhibited accelerated
cell death upon IL-3 deprivation.

8.   **Bcl-Y expression counteracts the survival function
      of Bcl-2.**

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The ability of Bcl-Y to promote cell death upon
withdrawal of IL-3 resembles the function of Bax, which has
been shown to increase the rate of apoptosis in FL5.12 cells
under the same conditions tested above (Oltvai, Z.N., et
20 al., Cell 74:609-619 (1993)). Bax was also reported to
counteract the enhanced survival provided by expression of
Bcl-2 in FL5.12 cells. To further compare the activity of
Bcl-Y to that of Bax, we asked whether expression of Bcl-Y
would similarly overcome the inhibition of apoptosis
25 afforded by expression of Bcl-2. FL5.12 cells were
transfected with an expression plasmid carrying human Bcl-2
which provides substantial protection against cell death
upon withdrawal of IL-3. To determine whether Bcl-Y could
overcome this function of Bcl-2, the cells were infected
30 with a recombinant retrovirus expression vector carrying the
HA-tagged Bcl-y protein and the gene for puromycin
resistance. For comparison, the FL5.12 cells expressing
Bcl-2 were separately infected with a retrovirus carrying
HA-tagged Bax, or a vector encoding puromycin resistance
35 only.

Pooled populations of puromycin-resistant cells expressing Bcl-2 (Bcl-2/puro population) were largely resistant to withdrawal of IL-3, reflecting the protective activity of Bcl-2 in these cells (Figure 10A). Viability of
5 the cell population expressing both Bcl-2 and HA-Bcl-y (Bcl-2/HA-Bcl-Y population) decreased faster, and to a greater extent, indicating that the protective function of Bcl-2 was partially overcome by Bcl-y in this cell population. As with Bcl-Y, expression of HA-Bax partly counteracted the
10 prolonged survival provided by Bcl-2 (Figure 10A). Thus, the effect of Bcl-Y on the survival of Bcl-2 expressing cells was as great or greater than that of Bax in this assay. Western blot analysis of these infected FL5.12 cell populations showed expression of HA-Bax and HA-Bcl-Y
15 proteins (Figure 10B). In addition, the increased sensitivity of cell populations expressing HA-Bcl-Y and HA-Bax to IL-3 withdrawal was not due to reduced levels of Bcl-2 (Figure 10B).

Additional experiments were performed by isolating
20 individual clones expressing Bcl-2 in combination with either HA-Bax or HA-Bcl-Y from the retrovirus-infected cell populations described above. Clones expressing the highest levels of exogenous HA-Bcl-Y or HA-Bax showed a more complete inhibition of Bcl-2 function than the cell
25 populations from which they were isolated (Figure 11). Viablity of Bcl-2/HA-Bcl-Y cl4 cells decreased more rapidly than a clone that did not express human Bcl-2 (neo, Figure 11A), indicating that Bcl-2 function in this clone was totally abrogated by Bcl-Y expression. Again, Western blots
30 demonstrated that similar levels of human Bcl-2 were expressed in the infected clones relative to the parental cell line (Figure 11B).

These results demonstrate that enforced expression of Bcl-Y can overcome the survival function of Bcl-2 and
35 restore sensitivity to signals that induce apoptosis (IL-3 withdrawal). There is experimental evidence to suggest that

certain tumor cells may require continued function of Bcl-2 (or related genes) for their survival ((L. Campos, et al., Blood 84:595-600 (1994); J.C. Reed, et al., Cancer Res. 50:6565 (1990)). Introduction of Bcl-y, or a functional
5 equivalent, into such tumor cells may restore sensitivity to signals that trigger apoptosis (i.e., deprivation of growth factors, treatment with chematherapeutic agents).

9. Bcl-Y interacts with Bcl-2 and Bcl-XL in vitro
10

It has been suggested that Bax may regulate Bcl-2 function through a specific protein/protein interaction (X.-M. Yin, et al., Nature 369:321-323 (1994)). The inhibition of Bcl-2 function by Bcl-y may therefore also be dependent
15 on a specific interaction between the two proteins. To test whether Bcl-y could interact with Bcl-2, glutathione S-transferase fusion proteins of Bcl-2 (GST-bcl-2) or its homolog Bcl-XL (GST- bcl-xl) were mixed with 35S-methionine-labeled HA-Bcl-Y generated by in vitro transcription/
20 translation. Resulting complexes were captured on glutathione-agarose and analyzed by SDS-PAGE. HA-Bcl-Y clearly co-precipitated with GST-Bcl-2 and to an even greater extent with GST-bcl-xl. No interaction was observed when HA-bcl-y was incubated with GST alone. Therefore, bcl-
25 y can specifically interact with bcl-2 and bcl-xl in vitro.

10. Chromosomal localization of the bcl-Y gene

Since multiple members of the Bcl-2 family may normally
30 be responsible for regulating cell death, a variety of mutational alterations could potentially disrupt this control and contribute to tumor development. For example, activation of Bcl-2 expression by a chromosomal translocation contributes to the onset of follicular
35 lymphoma. Analogous oncogenic lesions that suppress apoptosis could be generated, in principle, by the loss of a

gene such as bcl-Y that opposes bcl-2 function, and/or
positively regulates cell death. In this respect, bcl-Y may
be viewed as a candidate tumor suppressor gene.

To analyze whether loss or mutation of bcl-y is a
recurrent event in certain human tumors, the chromosomal
location of the bcl-Y gene was determined. A fragment of
human genomic DNA encompassing bcl-Y was isolated by
screening a P1 phage library (Genomic Systems, Inc.). The
bcl-Y genomic clone was used as a probe for fluorescent *in
situ* hybridation to metaphase chromosomes (BIOS
Laboratories). This analysis revealed that the bcl-Y
genomic locus maps to the short arm of human chromosome 6
(6p21.3). Abnormalities of 6p21 have been detected in
uterine leiomyomas (J. Mark, et al., Cancer Genet.
Cytogenet. 44:1 (1990)), astrocytomas (B.C. Liang, et al.,
Neurol. 44:533-536), benign lipomas (N. Mandahl, et al.,
Hum. Genet. 79:203-208), ovarian cancers (W. Cliby, et al.,
Cancer Res. 53:2393-2398), and endometrial polyps (P.D. Cin,
et al., Gynec. Oncol. 46:393-396). A large number of
diseases have been shown to be associated with the MHC
region, which also maps to 6p21 (J.L. Tiwari, and P.I.
Terasaki, HLA and Disease Associations. New York: Springer-
Verlag (1985)).

All publications mentioned in this specification are
indicative of the level of the skill of those skilled in the
art to which this invention pertains. All publications are
herein incorporated by reference, to the same extent as if
each individual publication was specifically and
individually indicated to be incorporated by reference.

While the invention has been described in connection
with the specific embodiments thereof, it will be understood
that it is capable of further modifications and this
application is intended to cover any variations, uses, or
adoptions of the invention following, in general, principles
of the invention and including such departures from the

present disclosure as come within known or customary
practice within the art to which the invention pertains and
as may be applied to the essential features hereinbefore set
forth and as follows in the scope of the appended claims.

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What is claimed is:

     1.   An isolated Bcl Y protein.

     2.   The isolated Bcl-Y protein of claim 1, wherein said protein is a human protein.

     3.   The isolated Bcl-Y protein of claim 2, wherein said protein consists essentially of the amino acid sequence as shown in Figure 4 [SEQ ID NO:16].

     4.   The isolated Bcl-Y protein of claim 2, wherein said protein is encoded by the nucleotide sequence as shown in Figure 4 [SEQ ID NO:17].

     5.   An isolated nucleotide sequence coding for Bcl-Y protein.

     6.   The isolated nucleotide sequence of claim 5, wherein said sequence is as shown in Figure 4 [SEQ ID NO:17].

     7.   The isolated nucleotide sequence of any one of claims 5 or 6, wherein said sequence comprises genomic DNA.

     8.   The isolated nucleotide sequence of any one of claims 5 or 6, wherein said sequence comprises cDNA.

     9.   The isolated nucleotide sequence of any one of claims 5 or 6, wherein said sequence comprise RNA.

     10.   An isolated recombinant DNA molecule consisting essentially of a nucleotide sequence that codes for Bcl-Y protein.

     11.   The isolated recombinant DNA molecule of claim 10, wherein said nucleotide sequence is as shown in Figure 4 [SEQ ID NO:17].

     12.   The isolated recombinant DNA molecule of claim 11, wherein said molecule is a vector.

13.　A vector consisting essentially of a recombinant DNA molecule encoding Bcl-Y protein, wherein said vector expresses a sequence that codes for Bcl-Y in said recombinant DNA molecule.

14.　A vector comprising a recombinant DNA molecule encoding Bcl-Y protein, wherein said vector expresses an antisense RNA of said recombinant molecule.

15.　The vector of claim 13, wherein said recombinant DNA molecule consists essentially of a nucleotide sequence as shown in Figure 4 [SEQ ID NO:17].

16.　A host cell transformed with the vector of any one of claims 13 or 15.

17.　The host cell of claim 11, wherein said host cell is a mammalian cell.

18.　A method for producing isolated Bcl-Y polypeptide, comprising:

(a)　constructing the vector of claim 13;

(b)　transforming a suitable host cell with said vector of Step (a);

(c)　culturing said host cell under conditions which allow the expression of said Bcl-Y polypeptide by said host cell; and

(d)　isolating said Bcl-Y polypeptide expressed by said host cell of Step (c);

wherein isolated substantially pure Bcl-Y polypeptide is produced.

19.　The method according to claim 18, wherein said host cell is a mammalian cell.

20.　An antibody raised against the Bcl-Y protein of claim 4.

21.   The antibody of claim 20, wherein said antibody is selected from the group consisting of: a polyclonal antibody, a monoclonal antibody, an anti-idiotypic antibody, and an anti- anti-idiotypic antibody.

5   22.   The antibody of claim 21, wherein said antibody is detectably labeled.

23.   The antibody of claim 22, wherein said antibody is detectably labeled with a detectable label selected from the group consisting of: a radio label, an enzyme label, a

10 co-factor label, a fluorescent label, a paramagnetic label, a chemiluminescent label, and a metal label.

24.   A detectably labeled nucleotide probe, comprising a first nucleotide sequence which is substantially complementary to a second nucleotide sequence that

15 specifically codes for the Bcl-Y protein of claim 4.

25.   A pharmaceutical composition comprising the Bcl-Y protein of claim 4 and a pharmaceutically acceptable carrier.

26.   A method for inducing apoptosis in a cell

20 comprising introducing into a nonapoptotic cell an amount of the Bcl-Y protein of claim 4 effective to induce apoptosis in said cell.

27.   The method of claim 26, wherein said Bcl-Y protein is introduced by expressing a nucleotide encoding Bcl-Y in

25 said nonapoptotic cell.

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```
          I
BCL-2   E E[L]F R D G . V N W G R I V A[F]F E F  G G V M C V E S V N R E M S P L
BCL-X   N E[L]F R D G . V N W G R I V A[F]F S F  G G A L C V E S V D K E M Q V L
BAX     A D M F S D G N F N W G R[V]V A L[F]Y F  A S K L V L K A L C T K V P E L
MCL-1   I H V F S D G V T N W G R I V T L I S F  G A F V A K H L K T I N Q E S C
A1      E K E F E D G I I N W G R I V T I[F]A F  G G V L L K K L P Q E Q I A L D
ASFV    T E[L]F K D[L]. I N W G R I[C]G F I V E[S A R M A K Y C K D A N N H L E
EBV     L E[I]F H R G D P S L[G R A L A]W M A W C M H A C R T L C C N Q S T P Y

                    N W G R(I/V/M)V

BD2:  5' AA(C/T)TCCCGN(A/C)GN(A/G)TNGT 3'
                                                              [SEQ ID NO. 13]


          II
V D N[T A L W M T E Y]L N R H[L]H T . . .[W I]Q D N G G W . . .[D]A F[V E]L Y  202  [SEQ ID NO. 6]
V S R[I A A W M A T Y]L N D H L E P . . .[W I]Q E N G G W . . .[D]T F[V E]L Y  195  [SEQ ID NO. 7]
I R T[L M G W T L D F]L R E R[L]L G . . .[W I]Q D Q G G W . . .[D]G[L]L S Y F  165  [SEQ ID NO. 8]
I E P L A E S I T D V L V R T K R D . . .[W L]V K Q[R G W]. . .[D]G[F]I K K F  319  [SEQ ID NO. 9]
V C A Y K Q V S S F V A E F I M N N T G E[W I]R Q N G G W E . . .[D]G F I K K F  148  [SEQ ID NO. 10]
S T V[I]T T A Y N F M K H N L[L P]. . .[W M]I S H G G Q . . .[E]E F L A F S  141  [SEQ ID NO. 11]
Y V V D L S V R G M L E A S E G L D G . . .[W]T[H Q Q G G W]. . . S T L[I][E]D N  157  [SEQ ID NO. 12]

                    G G W(D/E)(A/G)F

BU3:  3' CCNCCNACCCTNC(C/G)NAA(A/G) 5'
                                                              [SEQ ID NO. 14]
```

FIG.1A

BCL-Y
BCL-2
BCL-X
BAX
MCL-1
A1
ASFV
EBV

[SEQ ID NO. 15]
202 [SEQ ID NO. 6]
195 [SEQ ID NO. 7]
165 [SEQ ID NO. 8]
319 [SEQ ID NO. 9]
148 [SEQ ID NO. 10]
141 [SEQ ID NO. 11]
157 [SEQ ID NO. 12]

FIG.1B

FIG. 2

FIG.3

TGAGCCACCC GGGTTGGGCC AGGATCCCGG CAGGCTGATC CCGTCCTCCA CTGAGACCTG   60

AAAAATGGCT TCGGGGCAAG GCCCAGGTCC TCCCAGGCAG GAGTGCGGAG AGCCTGCCCT   120
    M   A   S   G   Q   G   P   G   P   P   R   Q   E   C   G   E   P   A   L

GCCCTCTGCT TCTGAGGAGC AGGTAGCCCA GGACACAGAG GAGGTTTTCC GCAGCTACGT   180
  P   S   A   S   E   E   Q   V   A   Q   D   T   E   E   V   F   R   S   Y   V

TTTTTACCGC CATCAGCAGG AACAGGAGGC TGAAGGGGTG GCTGCCCCTG CCGACCCAGA   240
  F   Y   R   H   Q   Q   E   Q   E   A   E   G   V   A   A   P   A   D   P   E

GATGGTCACC TTACCTCTGC AACCTAGCAG CACCATGGGG CAGGTGGGAC GGCAGCTCGC   300
  M   V   T   L   P   L   Q   P   S   S   T   M   G   Q   V   G   R   Q   L   A

CATCATCGGG GACGACATCA ACCGACGCTA TGACTCAGAG TTCCAGACCA TGTTGCAGCA   360
  I   I   G   D   D   I   N   R   R   Y   D   S   E   F   Q   T   M   L   Q   H

CCTGCAGCCC ACGGCAGAGA ATGCCTATGA GTACTTCACC AAGATTGCCA CCAGCCTGTT   420
  L   Q   P   T   A   E   N   A   Y   E   Y   F   T   K   I   A   T   S   L   F

TGAGAGTGGC ATCAATTGGG GCCGTGTGGT GGCTCTTCTG GGCTTCGGCT ACCGTCTGGC   480
  E   S   G   I   N   W   G   R   V   V   A   L   L   G   F   G   Y   R   L   A

CCTACACGTC TACCAGCATG GCCTGACTGG CTTCCTAGGC CAGGTGACCC GCTTCGTGGT   540
  L   H   V   Y   Q   H   G   L   T   G   F   L   G   Q   V   T   R   F   V   V

CGACTTCATG CTGCATCACT GCATTGCCCG GTGGATTGCA CAGAGGGGTG GCTGGGTGGC   600
  D   F   M   L   H   H   C   I   A   R   W   I   A   Q   R   G   G   W   V   A

AGCCCTGAAC TTGGGCAATG GTCCCATCCT GAACGTGCTG GTGGTTCTGG GTGTGGTTCT   660
  A   L   N   L   G   N   G   P   I   L   N   V   L   V   V   L   G   V   V   L

GTTGGGCCAG TTTGTGGTAC GAAGATTCTT CAAATCATGA   700
  L   G   Q   F   V   V   R   R   F   F   K   S   •   [SEQ ID NO. 16]

FIG.4

```
                                    CTCCCAAGGG TGCCCTTTGG       720
GTCCCGGTTC AGACCCCTGC CTGGACTTAA GCGAAGTCTT TGCCTTCTCT GTTCCCTTGC  780

AGGGGTCCCC CCTCAAGAGT ACAGAAGCTT TAGCAAGTGT GCACTCCAGC TTCGGAGGGC  840

CCCTGCGTGG GGGCCAGTCA GGCTGCAGAG GCACCTCAAC ATTGCATGGT GCTAGTGGGC  900

CCTCTCTCTG GGCCCAGGGG CTGTGGCCGT CTCCTCCCTC AGCTCTCTGG GACCTCCTTA  960

GCCCTGTCTG CTAGGCGCTG GGGAGACTGA TAACTTGGGG AGGCAAGAGA CTGGGAGCCA  1020

CTTCTCCCCA GAAAGTGTTT AACGGTTTTA GCTTTTTATA ATACCCTTGT GAGAGCCCAT  1080

TCCCACCATT CTACCTGAGG CCAGGACGTC TGGGGTGTGG GGATTGGTGG GTCTATGTTC  1140

CCCAGGATTC AGCTATTCTG GAAGATCAGC ACCCTAAGAG ATGGGACTAG GACCTGAGCC  1200

TGGTCCTGGC CGTCCCTAAG CATGTGTCCC AGGAGCAGGA CCTACTAGGA GAGGGCGGCC  1260

AAGGTCCTGC TCAACTCTAC CCCTGCTCCC ATTCCTCCCT CCGGCCATAC TGCCTTTGCA  1320

GTTGGACTCT CAGGGATTCT GGGCTTGGGG TGTGGGGTGG GGTGGAGTCG CAGACCAGAG  1380

CTGTCTGAAC TCACGTGTCA GAAGCCTCCA AGCCTGCCTC CCAAGGTCCT CTCAGTTCTC  1440

TCCCTTCCTC TCTCCTTATA GACACTTGCT CCCAACCCAT TCACTACAGG TGAAGGCTCT  1500

CACCCCATCC CTGGGGGCCT TGGGTGAGTG GCCTGCTAAG GCTCCTCCTT GCCCAGACTA  1560

CAGGGCTTAG GACTTGGTTT GTTATATCAG GGAAAAGGAG TAGGGAGTTC ATCTGGAGGG  1620

TTCTAAGTGG GAGAAGGACT ATCAACACCA CTAGGAATCC CAGAGGTGGG ATCCTCCCTC  1680

ATGGCTCTGG CACAGTGTAA TCCAGGGGTG TAGATGGGGG AACTGTGAAT ACTTGAACTC  1740

TGTTCCCCCA CCCTCCATGC TCCTCACCTG TCTAGGTCTC CTCAGGGTGG GGGGTGACAG  1800

TGCCTTCTCT ATTGGGCACA GCCTAGGGTC TTGGGGGTCA GGGGGGAGAA GTTCTTGATT  1860

CAGCCAAATG CAGGGAGGGG AGGCAGATGG AGCCCATAGG CCACCCCCTA TCCTCTGAGT  1920

GTTTGGAAAT AAACTGTGCA ATCCCCTCAC CCTGAAAAAA AAAAAAAA [SEQ ID NO. 17]  1968
```

FIG.4(cont.)

BCL-2 60 A A S R D P V A R T S P L Q T P A A P G [SEQ ID NO. 18]
BCL-Y 53 A A P A D P E M V I P L Q P S S T W G [SEQ ID NO. 19]

BCL-2 101 G D D F S R R Y R G D F A E M S S Q L H L T P F T A R G R F A T V V E E L F R D G V N W G R I V A F F E F G [SEQ ID NO. 20]
BCL-Y   82 G D D I N R R Y D S E F Q T M L Q H L O P U A E N A Y E Y F T K I I A T S L F E S G I N W G R V V A L L G F G [SEQ ID NO. 21]

BCL-2 181 L N R H L H T W I Q D N G G W D A F V E L [SEQ ID NO. 22]
BCL-Y 163 L H H C I A R W I A Q R G G W V A A L N L [SEQ ID NO. 23]

FIG.5

I

		Sequence	
BCL-2	93	VHLALRQAGDDFSRRYRGDFAEMSSQLHLTPFTARGRFATVVEELFRDG.	[SEQ ID NO. 24]
BCL-Y	74	VCRQLAIIGDDINRRYDSEFQTMLQHLQPTAENAYEYFTKIATSLFESG.	[SEQ ID NO. 25]
BCL-X	86	VKQALREAGDEFELRYRRAFSDLTSQLHITPGTAYQSFEQVVNELFRDG.	[SEQ ID NO. 26]
BAX	59	LSECLKRIGDELDSN.MELQRMIAAVD.IDS.PREVFFRVAADMFSDGN	[SEQ ID NO. 27]
MCL-1	209	ALETLRRVGDGVQRNHETVFQGMLRKLDIKNEDDVKSLSRVMIHVFSDGV	[SEQ ID NO. 28]
AI	34	CRVLQRVAFSVQKEVEKNLKSYLDDFHVESIDTARIIFNQVMEKEFEDGI	[SEQ ID NO. 29]
ASFV	38	QIKKILTYYDECLNK.QVTITFSLTNAQEIKTQFTGVVTELFKDL.	[SEQ ID NO. 30]
EBV	46	VLRYHVLLEEIIERNSETETWNRFITHTEHVDLDFNSVFLEIFHRGD	[SEQ ID NO. 31]

II

		Sequence	
BCL-2	142	VNWGRIVAFFEFGGVMCVESVNREMSPLVDNIALWMTEYLNRHLHT...	[SEQ ID NO. 24]
BCL-Y	123	INWGRVVALLGFGYRLALHYYQHGLTGFLGQVIRFVVDFMLHCIAR...	[SEQ ID NO. 25]
BCL-X	135	VNWGRIVAFFSFGGALCVESVDKEMQVLVSRIAAWMATYLNDHLEP...	[SEQ ID NO. 26]
BAX	105	FNWGRVVALFYFASKLVLKALCTKVPELIRTIMGWTLDFLRERLLG...	[SEQ ID NO. 27]
MCL-1	259	TNWGRIVTLISFGAFVAKHLKTINQESCIEPLAESITDVLVRTKRD.	[SEQ ID NO. 28]
AI	84	INWGRIVTIFAFGGVLKKLPOEQIALDVCAYKQVSSFVAEFIMNNTGE	[SEQ ID NO. 29]
ASFV	82	INWGRICGFIVESARWAKYCKDAN.NHLESTVITTAYNFMKHNLLP.	[SEQ ID NO. 30]
EBV	96	PSLGRALAWMAWCMHACRTLCCNQSTPYYVVDLSVRGMLEASEGLDG..	[SEQ ID NO. 31]

|		Sequence	
BCL-2	188	WIQDNGGWDAEIVELY.GPSMRPLFDF.SWLSLKTLLSLALVGACITLGAYLSHK	[SEQ ID NO. 24]
BCL-Y	170	WIAQRGGWVAALNLGNGP..........ILNVLVLGVVLLGQFVVRRFFKS[SEQ ID NO. 25]
BCL-X	181	WIQENGGWDTFVELYGNNAAAESRKGQERFNRWFLTGMIVAGVVLLGSLFSRK[SEQ ID NO. 26]
BAX	151	WIQDQGGWDGLLSYFGTPT...........WQTVTIFVAGVLTASLLTIWKKMG[SEQ ID NO. 27]
MCL-1	305	WLVKQRGWDGFIVEFFHVEDLEGGI......RNVLLAFAGVAGVGAGLAYLIR[SEQ ID NO. 28]
AI	133	WIRONGGWEDGFIKKFEP........KSGWLIFLQMTGQIWEMLFLLK[SEQ ID NO. 29]
ASFV	127	WMISHGGQEEFLAFSLHSDIYSVIFNIKYFLSKFCNHMFLRSCVOLLRNCNLI[SEQ ID NO. 30]
EBV	143	WIHQQGGWSTLIEDNIPGS.......RFSWTLFLAGLTLSLLVICSYLFISRGRH[SEQ ID NO. 31]

FIG.6
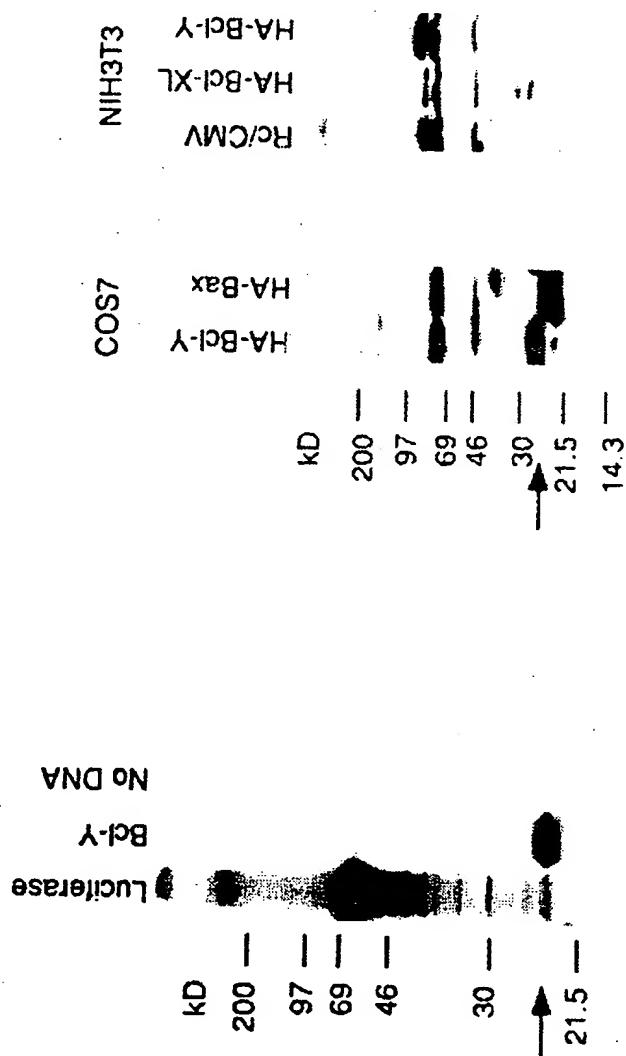
FIG. 7B

FIG.7A
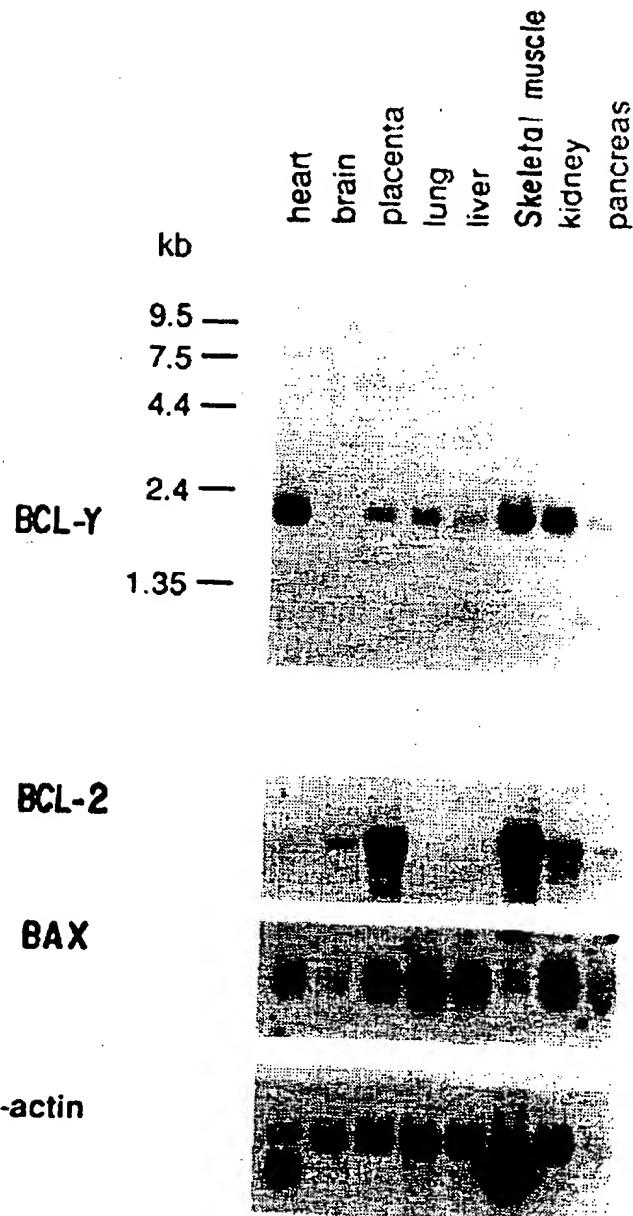
FIG. 8
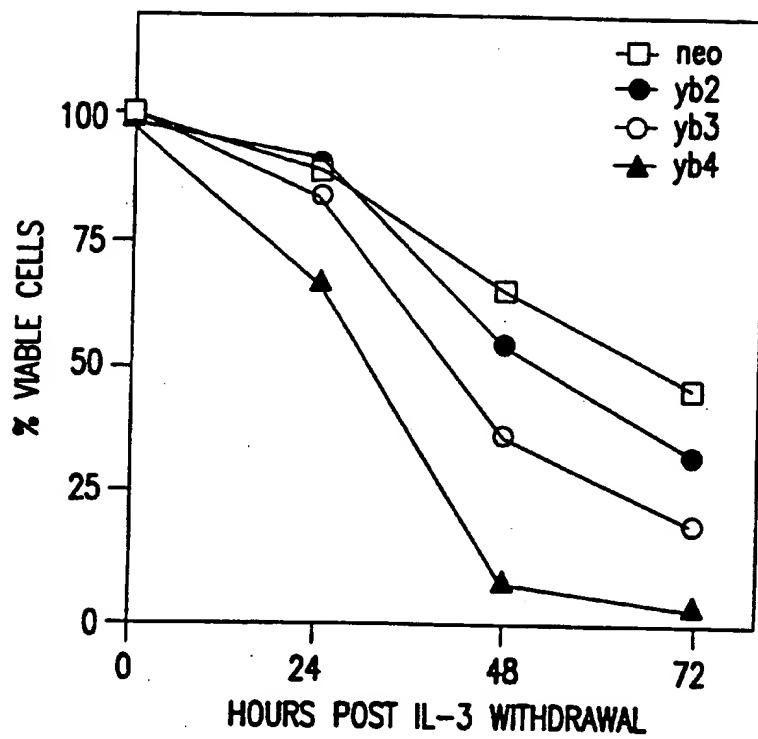
FIG.9A

12 / 16

FIG. 9B

13 / 16

FIG.10A

14 /16

HA-Bcl-y
HA-Bax

Neo/Puro
Bcl-2/Puro
Bcl-2/HA-Bax
Bcl-2/HA-Bcl-y

Bcl-2

Neo/Puro
Bcl-2/Puro
Bcl-2/HA-Bax
Bcl-2/HA-Bcl-y
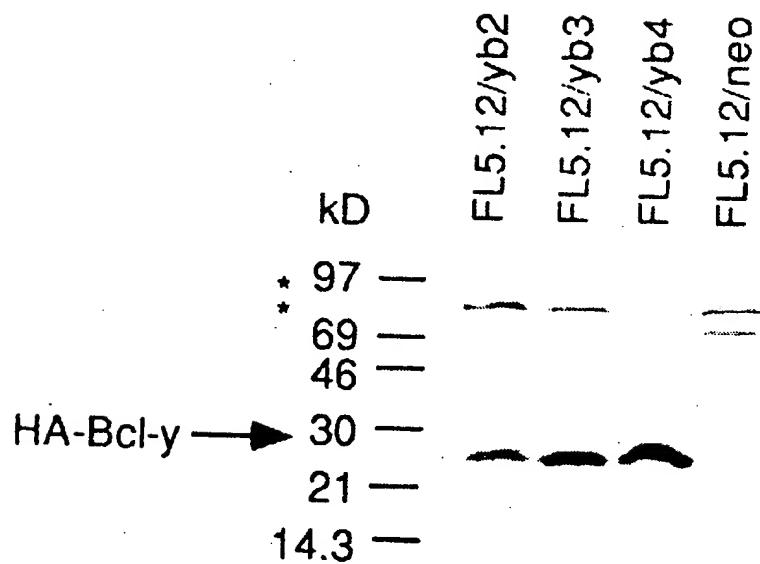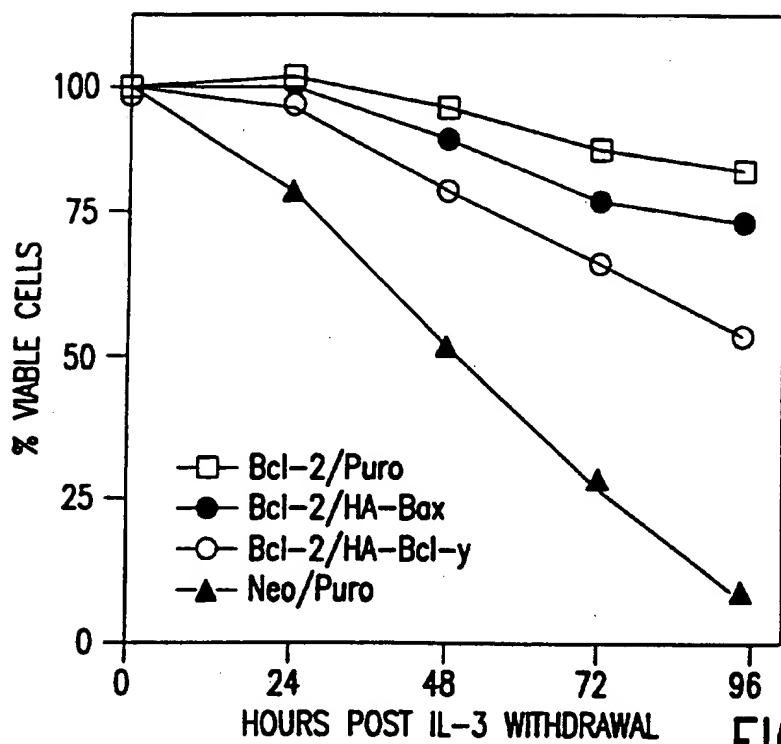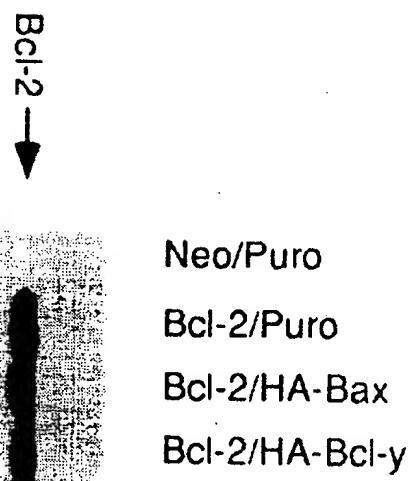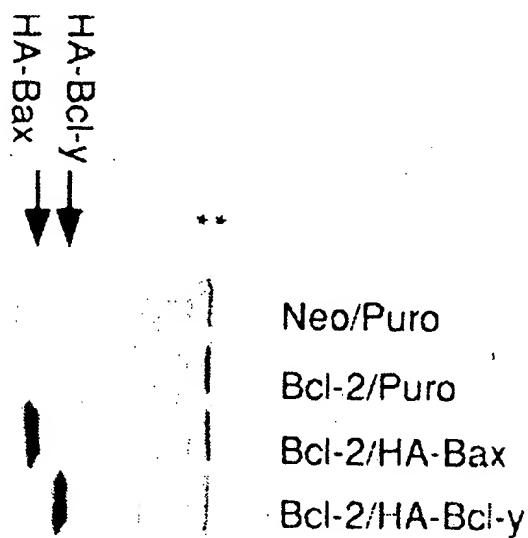
FIG. 10B

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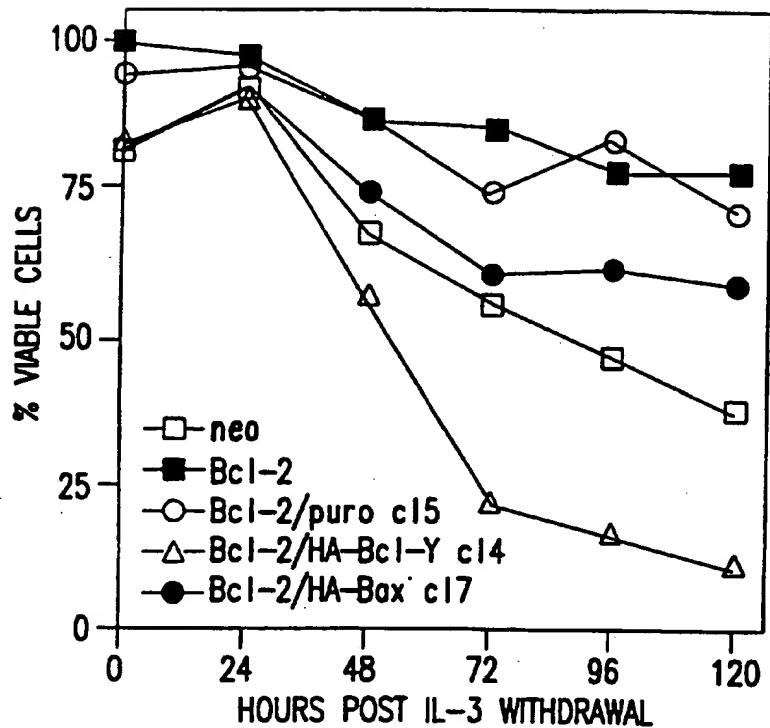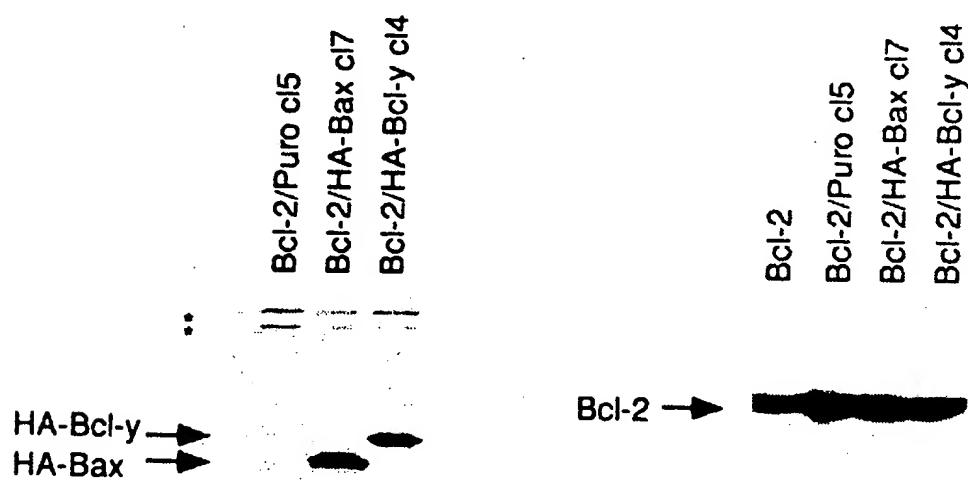
FIG.11A

FIG. 11B

INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :530/350+, 387.1; 536/23.1; 435/70.3, 240.1, 252.3; 514/12, 44
According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350+, 387.1; 536/23.1; 435/70.3, 240.1, 252.3; 514/12, 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Chemical Abstracts, Biosis

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Proceedings of the National Academy of Sciences USA, Volume 83, issued July 1986, Y. Tsujimoto et al, "Analysis of the Structure, Transcripts and Protein Products of *bcl-2*, the Gene Involved in Human Follicular Lymphoma", pages 5214-5218, see especially page 5216, figure 3 and page 5217, figure 4, and "Discussion".	1-19
X	Cancer Research, Volume 53, Number 14, issued 15 July 1993, T. C. Fisher et al, "bcl-2 Modulation of Apoptosis Induced by Anticancer Drugs: Resistance to Thymidylate Stress is Independent of Classical Resistance Pathways", pages 3321-3326, see especially page 3325, column 2, paragraph 1 to page 3326, line 8.	25-27

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 OCTOBER 1995	**22 NOV 1995**

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Deborah Crouch, Ph.D.
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Form PCT/ISA/210 (second sheet)(July 1992)

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Analytical Biochemistry, Volume 205, issued 1992, J.C. Reed et al, "A Strategy for Generating Monoclonal Antibodies Against Recombinant Baculovirus-Produced Proteins: Application to the Bcl-2 Oncoprotein", pages 70-76, see especially page 73, column 2, paragraph 1.	20-24

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07K 17/00, 16/00; C07H 21/02; C12N 1/20, 5/00; C12P 21/04; A61K 38/00, 31/70